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A Publication of the American Veterinary Medical Association

Volume XVIII APRIL, 1957 No. 67

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Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Additional entry as Second-Class Matter at the Post Office at Champaign, Illinois, approved February 17, 1953. Reproduction of any part of this publication is prohibited, unless special permission is given by the publishers and the authors. Subscription price: $5.00 per year for United States, Canada and Pan American Countries, $5.50; foreign countries, $6.00. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $3.00 per year added to the annual dues of $15.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

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VOL. XVIII APRIL, 1957 No. 67

Experimental Studies of the Innervation of the Abdominal Wall of Cattle

J. P. ARNOLD, D.V.M., Ph.D., and R. L. KITCHELL, D.V.M., Ph.D.

St. Paul, Minnesota

PARAVERTEBRAL ANESTHESIA in cattle was introduced by Farquharson [10] in 1942. It gained popularity with a number of veterinary surgeons but, because of unsatisfactory results, a large number of veterinarians have returned to local infiltration as a means of anesthetizing the abdominal wall. Others continue to use paravertebral anesthesia and, if necessary, supplement it with local infiltration.

The advantages of paravertebral or segmental anesthesia for surgical procedures involving the lateral abdominal wall of cattle and the inconsistent results associated with the technique described by Farquharson indicated to the authors the need for additional fundamental research on this problem.

The studies were divided into three parts, namely: (1) gross anatomic dissections, (2) experimental transections of various spinal nerves or their components, and (3) clinical trials. The clinical trials will be described in a subsequent paper.

From the Divisions of Veterinary Surgery and Radiology (Arnold) and Veterinary Anatomy (Kitchell), School of Veterinary Medicine, Institute of Agriculture, University of Minnesota, St. Paul.

Based on a thesis submitted by the senior author to the Graduate School of the University of Minnesota, in partial fulfillment of the requirements for the Ph.D. degree.

Approved for publication as scientific journal series paper 3589, Minnesota Agricultural Experiment Station, St. Paul.

The authors gratefully acknowledge the advice and assistance rendered by M. H. Roepke, Ph.D., the technical assistance of Miss Margie Slints, and the drawings and sketches by Donald Johnson of the Department of Medical Illustrations.

The study was supported, in part, by a research grant from Sharp and Dohme, Inc., Division of Merck and Co., West Point, Pa.

I) GROSS ANATOMY

A) REVIEW OF LITERATURE

Few references can be found in the literature which deal specifically with the anatomic distribution of the thoracic and lumbar spinal nerves in cattle. The most noteworthy articles have been those contributed by St. Clair,[20] Farquharson,[10] and Emmerson.[9] Additional information can be obtained from textbooks on gross anatomy or guides, such as those of Montane and Bourdelle,[23] Martin and Schauder,[22] Habel,[12] and Peterson [24] on the anatomy of cattle. Textbooks on comparative anatomy, such as Ellenberger and Baum [8] and Sisson and Grossman,[28] emphasize the horse and only compare the cow to the horse.

B) METHOD OF PROCEDURE

Twenty-two animals were used in the part of the study on gross anatomy. Eleven of these animals were embalmed cadavers used for dissection by students in the gross anatomy laboratory. They consisted of 8 cows varying in weight from 700 to 1,100 lb., 2 bulls weighing 800 lb. each, and 1 bull weighing 300 lb. The dissection of the last few thoracic and the lumbar nerves in these animals was performed by students with special instructions from the authors.

The remaining 11 animals were secured from the postmortem laboratory. They ranged in weight from 150 to 550 lb. These animals were dissected as fresh specimens.

In all instances, each animal was given a number and the observations were recorded by the authors for each animal. Photographs were taken of some animals during the course of the dissection and of all animals at the conclusion of the dissection.

C) OBSERVATIONS

The courses of the spinal nerves are described somewhat in detail. Other workers have followed the courses of these nerves but failed to describe them adequately

with regard to innervation of the abdominal wall.

The eleventh, twelfth, and thirteenth thoracic and the lumbar spinal nerves emerge from their respective intervertebral foramina and, after passing a short distance between the multifidus dorsi muscle dorsally and the quadratus lumborum muscle located ventrally, divide into dorsal and ventral branches (fig. 1). The dorsal branch of the spinal nerve turns dorsally and gives off dorsomedial branches which enter the multifidus dorsi and longissimus dorsi muscles.

The dorsal branch continues laterally as the dorsolateral nerve. The dorsolateral branches of the eleventh and twelfth thoracic spinal nerves pass between the longissimus dorsi and the longissimus costarum muscles and give off branches to each (fig. 1). The dorsolateral branch crosses over the rib posterior to it at about the level of the lateral edge of the transverse process of the second lumbar vertebra. Following this, it enters the serratus dorsalis posterior muscle and emerges from the lumbodorsal fascia accompanied by blood vessels (fig. 2). Previous to its emergence from the lumbodorsal fascia, the nerve divides into two branches. One branch, along with a larger artery and vein, turns dorsally and enters the skin. The other branch proceeds ventrally with small blood vessels on the surface of the serratus dorsalis posterior muscle and across the origin of the external abdominal oblique muscle. The branch which proceeds ventrally ramifies in the skin at about the level of the tuber coxae.

The dorsal branch of the thirteenth thoracic spinal nerve passes between the longissimus dorsi and intertransversales lumborum muscles and then continues between the longissimus costarum muscles to emerge through the fibers of the serratus dorsalis posterior muscle and perforate the lumbodorsal fascia (fig. 1 and 2). The point of emergence is at about the same level as the costochondral junction of the last rib. The branch passing dorsally is accompanied by the larger branch of the associated artery and vein and goes in a posterolateral direction on the surface of the internal abdominal oblique muscle to ramify in the skin of the flank slightly ventral to the level of the tuber coxae.

The dorsolateral branches of the first to fifth lumbar spinal nerves, accompanied by a branch of their respective lumbar arteries, pass under the longissimus dorsi muscle and on the surface of the strong fascia of the intertransversales lumborum muscle. In the authors' experience, this fascia is heavy enough to prevent anesthetic agents

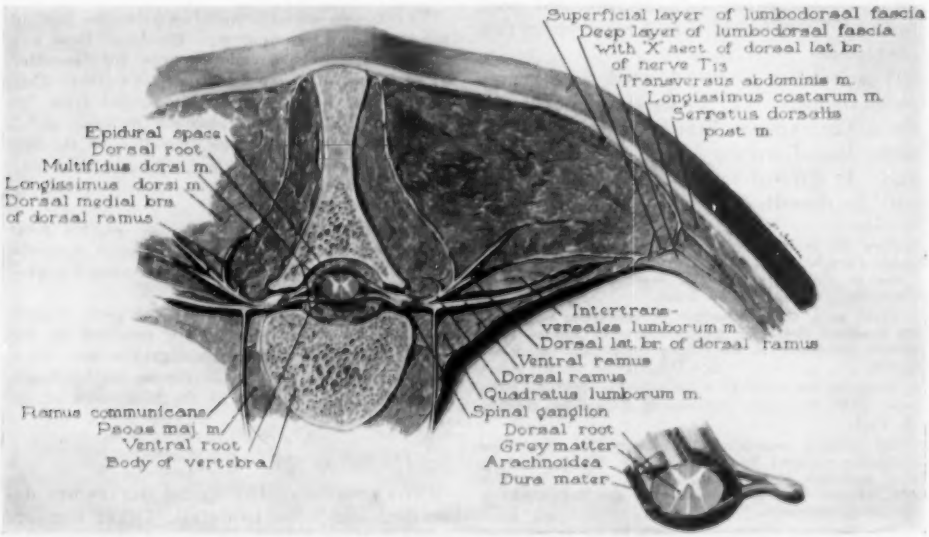

Fig. 1—Transverse section through vertebral column showing relations of spinal nerve at the level of the first lumbar spinal nerve.

from diffusing through it. For varying distances, they follow a course along the posterior border of the transverse process of their respective vertebrae and then pass over the lateral edge of the transverse process posterior to them. The first three dorsolateral branches emerge from the lumbodorsal fascia at about the level of the tuber coxae and the last three emerge nearer the dorsal midline with the sixth emerging closest to it. The dorsolateral branch of the first lumbar spinal nerve may emerge between the longissimus dorsi and the longissimus costarum muscles or it may perforate the longissimus dorsi muscle. In some instances, it divides within the longissimus dorsi muscle and the branches pierce the lumbodorsal fascia at different points. The dorsolateral branches of the second and third lumbar spinal nerves may also divide before emerging from the lumbodorsal fascia and follow an independent course to the skin. The twig of the dorsolateral branch of the third lumbar spinal nerve follows a ventrally directed course over the internal abdominal oblique muscle to the external abdominal oblique muscle and then, on its surface, anterior to the prefemoral lymph nodes where it ramifies in the skin. It follows a course near the borders of the gluteus medius and the biceps femoris muscles and terminates in the skin at about the level of the tuber coxae.

The ventral branch of each of the eleventh and twelfth thoracic spinal nerves continues laterally between the quadratus lumborum and longissimus dorsi muscles and later passes between the internal and external intercostal muscles. Each nerve is accompanied by a large intercostal artery and vein as it runs along the posterior border of its respective rib. The nerve divides into ventrolateral and ventromedial branches at the level of the tuber coxae. The branches proceed together for a distance and then the ventrolateral branch perforates the external intercostal muscle and runs between it and the external abdominal oblique muscle. The nerve passes in a posteroventral direction, perforates the external abdominal oblique muscle, and goes between the external abdominal oblique muscle and the cutaneous trunci muscle to ramify in the skin. The ventromedial branch proceeds between the intercostal muscles and then passes on the surface of the transverse abdominis muscle. It continues in a ventroposterior direction and goes through the rectus abdominis

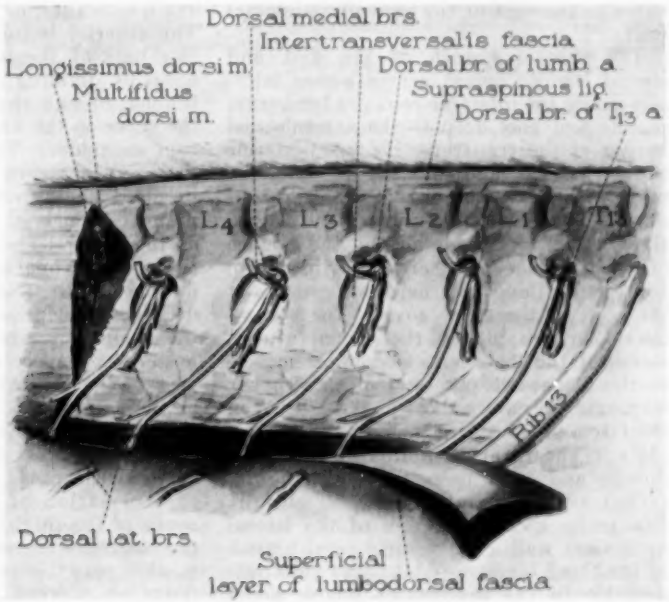

Fig. 2—Dorsolateral view of lumbar area of bull showing dorsal branches of spinal nerves.

muscle and the aponeuroses of the internal and external abdominal oblique muscles, through the cutaneous trunci muscle, to end in the skin anterior to the umbilicus.

The ventral branch of the thirteenth thoracic spinal nerve continues laterally between the quadratus lumborum and the intertransversales lumborum muscles. It leaves the posterior border of the transverse process of the thirteenth thoracic vertebra and follows a course which brings it deep to the anterolateral edge of the first lumbar vertebra. It runs under the longissimus costarum where it divides into ventrolateral and ventromedial branches. The ventrolateral branch passes through the transverse abdominis muscle and runs between it and the internal abdominal oblique muscle for a short distance. It then passes through the internal and external abdominal oblique muscles, through the cutaneous trunci muscle, and ends in the skin of the lateral abdominal wall. The ventromedial branch passes through the transverse abdominis muscle with the ventrolateral branch and continues on the surface of the transverse abdominis muscle. It runs in a posteroventral direction through the rectus abdominis muscle and the aponeuroses of the internal and external abdominal oblique muscles and terminates in the skin of the ventral abdominal wall.

The ventral branch of the first and second lumbar spinal nerves passes laterally under the intertransversales lumborum muscle and goes deep to the anterolateral border of the transverse process posterior to it. The second lumbar spinal nerve may supply a branch to the inguinal nerve. The nerve passes under the longissimus costarum muscle and on the surface of the peritoneum where it divides into ventrolateral and ventromedial branches at the level of the tuber coxae. The ventrolateral branch pierces the fascial attachments of the transverse abdominis muscle to the transverse processes of the lumbar vertebrae. It runs a short distance between the fascia of the transverse abdominis muscle and the internal abdominal oblique muscle and then passes through the internal and external abdominal oblique muscles to end in the skin of the lateral abdominal wall. The ventrolateral branch of the third lumbar spinal nerve runs just anterior to the prefemoral lymph nodes and ends in the skin of the flank. The ventromedial branch of both spinal nerves passes through the transverse abdominis muscle, goes under the rectus abdominis muscle, then passes through this muscle and the aponeuroses of the internal and external abdominal oblique muscles, and terminates in the skin of the ventral abdominal wall.

The ventral branch of the third lumbar spinal nerve passes ventrally between the psoas major and minor muscles in a posterior direction. Fibers are distributed to the inguinal nerve and the lateral cutaneous nerve of the thigh.

The inguinal nerve, which is always formed by a part of the ventral branch of the third lumbar spinal nerve, often contains components from the ventral branches of the second and fourth lumbar spinal nerves. It continues between the psoas muscles in the posteroventral direction. Anterior and posterior branches of the inguinal nerve are formed. These branches proceed posteriorly, the anterior branch goes beneath the circumflex iliac artery and the posterior branch goes over the artery. They run ventrally on the external surface of the peritoneum to become associated with the iliac vessels. The two branches join just before reaching the internal inguinal ring and they separate again into anterior and posterior branches. The anterior branch emerges at the anterior part of the inguinal ring. It terminates by dividing into two branches. One branch goes to the fold of the flank and the other to the anterior part of the scrotum or udder. The posterior branch of the inguinal nerve as it emerges from the inguinal ring gives off one or two small branches which go to the anterior quarter of the udder. The main branch passes to the posterior quarter. This agrees with the findings of St. Clair [29] who gives a more detailed description of the innervation of the mammary gland. In the male, the posterior branch of the inguinal nerve goes to the central and posterior surfaces of the scrotum.

Ventral branches of the fourth lumbar spinal nerve pass in a posterior direction between the psoas muscles and enter into the formation of the lateral cutaneous nerve of the thigh, the femoral nerve, and the obturator nerve. As mentioned above, it also may contribute to the inguinal nerve.

The lateral cutaneous nerve of the thigh

passes in a posterior direction between the psoas muscles and across the surface of the peritoneum toward the anterior border of the tuber coxae. Here, it perforates the external abdominal oblique muscle and goes under the prefemoral lymph nodes to reach the skin in the region of the thigh.

II) Experimental Studies of Sensory Innervation of the Lateral Abdominal Wall of Cattle

A) Determination of Cutaneous and Peritoneal Distribution of Spinal Nerves T13, L1, L2, and L3

1) *Review of Literature.*—The area of cutaneous distribution of an individual spinal nerve is called a dermatome. Dermatomes have been investigated in the frog,[7, 31] cat,[1, 2, 17, 23] dog,[3, 26, 35, 37] monkey,[17, 21, 33] man, [5, 13, 14, 15] pigeon, [16, 34] fish,[27] and the lizard.[36] No reports have been found in the literature describing dermatomes of the bovine species.

Several methods have been used to isolate a particular dermatome. These methods may be grouped under seven main headings.

a) Sherrington's Method of Remaining Sensitivity.—This method probably originated with Eckhard[7] who reported briefly on the cutaneous distribution of the last four sensory roots in the frog. He cut all of the sensory roots in the area except that of the spinal nerve being studied. Sherrington[23, 32] utilized this method extensively in the frog, cat, and monkey. This worker cut the dorsal roots of two or three nerves on each side of the dorsal root of the spinal nerve being studied.

b) Anatomical Method of Herringham and Bolk.—Herringham[15] and Bolk,[5] working independently, developed this method. These workers painstakingly followed the fibers of a dorsal root into mixed nerves and through plexuses to the skin. Their method has the disadvantage that it is not possible to follow accurately the fine ramifications of the nerve fibers.

c) Strychnine Method of Dusser de Barenne.—Dusser de Barenne[6] applied strychnine solution to the exposed dorsal root of a spinal nerve. This caused the area supplied by the nerve to become hyperesthetic. The strychnine method has an advantage over Sherrington's method in that several dermatomes can be mapped in the same animal.

d) Method of Head.—Head and Campbell[14] utilized clinical cases of herpes zoster in man to map dermatomes. In this disease, cutaneous eruptions appear in the area supplied by fibers from the involved dorsal root ganglion or ganglia.

e) Method of Forester.—Forester[11] outlined dermatomes in man following surgical procedures during which dorsal roots were transected for the relief of pain. In some instances, he applied faradic stimulation to the proximal end of the peripheral stump and mapped the area of vasodilation which resulted from the stimulation. In other cases, he waited until the patient had recovered from surgery and then mapped the desensitized area. He considered the border of sensation present just above a desensitized area as the inferior border of the dermatome of the first intact dorsal root superior to the transected root. Likewise, the similar area inferiorly was considered the superior border of the first intact dorsal root inferior to the transected dorsal root.

f) Spinal Transection Method of Rossi.—Rossi,[20] using dogs, made a transverse cut through the spinal cord at the posterior edge of the segment being studied. He considered the posterior border of the sensitive area as the posterior extent of the dermatome of the last intact root.

g) Oscillographic Method.—The oscillographic method has been utilized by Kuhn[17] to map the lumbosacral dermatomes in the cat and the monkey. This method involves transection of the dorsal root proximal to the dorsal root ganglion and recording from the peripheral portion of the root between the cut end and the ganglion. The area considered to be innervated by the peripheral ramifications of the root is stimulated by means of a brush attached to an electromagnet. The sweep of the oscillograph is synchronized with the electromagnet. If the area of the skin tested is innervated by the root from which the recordings are made, an action potential will appear on the beam of the oscillograph.

For the purpose of illustrating the location and extent of a number of dermatomes on an experimental animal and for determining the amount of overlap between the adjacent dermatomes, various techniques were developed by different investigators for the purpose of transferring the extent of an isolated dermatome to an outline of an animal. One method is to transfer the outline of the dermatomes to plaster cast models[21] and sketched outlines of animals.[1, 2, 33] Photographing the animals has also been used.[16, 27, 29, 30] Rossi[20] photographed the living animal, destroyed and skinned it, and then photographed the stretched skin. A composite was then prepared from the two photographs.

The overlapping of dermatomes of adjacent dorsal roots has been the subject of investigation since Eckhard[7] briefly mentioned the cutaneous distribution of the last four spinal roots in the frog and recorded that two adjacent dermatomes partly overlap each other. Herringham[15] and Bolk,[5] as the result of anatomic dissections, found each dermatome to be separate with no overlapping. Sherrington[21, 32] reported that, in the monkey, a given dermatome would overlap up to about one half of the field of each of the adjacent dermatomes. Van Rijnberk[27] and Ten Cate and Waterman[29] found a similar type of overlapping in the dog. They reported the overlapping to be about one third of each adjacent dermatome. De Boer[1, 2] found that a dermatome in the cat would overlap two thirds of the adjacent dermatomes in certain areas. Van Trigt[30] mapped the

dermatomes in the lizard and found the overlapping to be two thirds of the adjacent dermatomes. Kaiser [18] reported the overlapping in the pigeon to be one half of the adjacent dermatomes.

There have been a number of investigations to determine the sensory innervation of the parietal peritoneum. Lennander [19, 20] called attention to the fact that the parietal peritoneum of man was sensitive to pain. He found that in an area extending from 4.5 cm. above the umbilicus to a point midway between the umbilicus and the symphysis pubis the parietal peritoneum was sensitive to stroking or stretching but not to thermal stimuli or to pressure. Ranström [28] made small incisions in the abdominal wall and palpated the parietal peritoneum through these incisions. Heavy pressure with his finger was not felt as such by the patient but at times resulted in colicky pain. Lewis and Kellgren [21] stimulated the parietal peritoneum of the cat. The animal was decapitated by Sherrington's method and incisions were made through the abdominal wall parallel to the large nerves. The parietal peritoneum was stroked with a blunt probe or a cotton applicator stick. They pressed firmly enough to raise the abdominal wall with each stroke. The posterior abdominal wall below the level of the kidneys gave little or no response. When the center of the diaphragm was stroked, there was no response but, when the peripheral part of the diaphragm was stroked, there were sharp twitches of the thoracic and abdominal muscles. The anterior and lateral parts of the abdominal cavity produced small responses in some areas and no response in others. Capps and Coleman,[4] using local anesthesia, introduced a trocar through the abdominal wall of man. A silver wire with a beaded end was inserted through the cannula and the peritoneum was scratched. They found that the parietal peritoneum was sensitive as far as they explored. They stimulated the parietal peritoneum as far inferiorly as the superior iliac spines. The parietal peritoneum was sensitive to strong pressure from a smooth bead and to light pressure of scratching with a rough end of the wire. The peritoneal covering of the diaphragm was sensitive to strong pressure or scratching. Shaw [26] emphasized that, under local anesthesia, pinching of sympathetic ganglia results in pain after an interval of time.

2) *Method of Procedure.*—General anesthesia was used on all of the animals. A 5 or 6 per cent solution of chloral hydrate when given intravenously proved to be the most satisfactory agent for the prolonged anesthesia needed in this type of surgery. Metropine®* was given to control salivation and reduce intestinal motility during surgery.

The operative area was clipped, scrubbed, dried, and a 70 per cent ethyl alcohol solution was then applied. A skin incision was made slightly lateral to the dorsal midline on the side where the dorsal rootlets were to be cut. The incision extended from the summit of the spinous process of the third lumbar vertebra anterior to the first vertebra whose rootlets were to be cut and extended about two vertebrae posterior to the last vertebra whose rootlets were to be cut. The lumbodorsal fascia was incised and the longissimus dorsi and multifidus dorsi muscles were separated by blunt dissection. Then, beginning at the second spinous process anterior to the first one whose rootlets were to be cut, the periosteum, along with the insertions of the multifidus muscles, was separated from the spinous processes using a periosteal elevator. The multifidus muscles were reflected laterally and the arches of the vertebrae were exposed. A rongeur was used to remove part of the arch and thus expose the dorsal rootlets, of the spinal nerve, which were to be cut. A suction apparatus was used to keep the operative area free of blood. The extradural fat was then removed to expose the dorsal rootlets. A small curved hemostat was used to elevate them and they were cut extradurally.

The dorsal rootlets of three adjacent spinal nerves anterior to the one whose dermatome was being determined and those of two or three adjacent spinal nerves posterior to it were cut. When the dermatome of third lumbar spinal nerve was being determined, only the dorsal rootlets of the fourth and fifth spinal nerves were cut posterior to it. This avoided the paralysis that resulted when the dorsal rootlets of the sixth spinal nerve were cut in addition to the fourth and fifth.

After the dorsal rootlets had been cut, gelfoam sponges were used to control hemorrhage in the vertebra where a portion of the arch had been removed. After placing aqueous penicillin in the incision, it was closed with interrupted mattress and simple continuous sutures of silk.

The aftercare consisted of administering periodic intramuscular injections of procaine penicillin and keeping the animal comfortable. Most of the animals were able to stand 24 hours after surgery. Of those which were unable to stand at this time, only 2 responded later. Animals which were unable to stand were discarded from the experiment since they did not respond properly to the testing procedures.

The area of remaining sensitivity was mapped after the initial swelling had subsided and the animal had become at ease with its surroundings. The mapping procedures were carried out in a room free of external noises, movements of people or objects, and insects such as flies. The test stimulus used was a prick or scratch with a sharp 18-gauge hypodermic needle. The area of sensation was mapped by moving the test stimulus from a nonsensitive area toward the sensitive area. When a response was elicited the point was marked with a water-soluble, showcard paint which contrasted with the color of the hair in the area. Later, the points were checked and, if shown to be in error, the color could easily be removed and the correct one marked. When a dermatome had been marked, the entire area was

* Metropine is the trade name of methyl atropine nitrate solution, R. J. Strassenburgh Co.

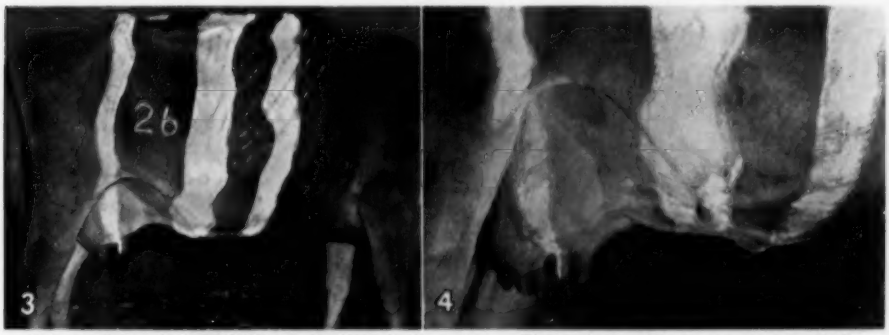

Fig. 3—Lateral view of thirteenth thoracic dermatome. The central painted area represents the isolated dermatome. The unpainted areas adjacent to it are nonsensitive due to transection of dorsal roots of T10, 11, 12, and L1, L2, and L3.

Fig. 4—Closer view of animal shown in figure 3.

painted with showeard paint and photographed. A medical artist then made composite drawings of each dermatome mapped. All the dermatomes mapped were drawn on an outline of an animal.

The area of desensitization of the parietal peritoneum was mapped on 4 animals after a dermatome had been mapped. A laparotomy, using local anesthesia, was performed on the opposite abdominal wall. A 6-inch incision was made through the abdominal wall in the region of the paralumbar fossa. The hand was passed through this incision and around the sublumbar attachments of the common mesentery and the rumen. In this way, the parietal peritoneum of the opposite abdominal wall could easily be stimulated. The peritoneum was scratched with the thumbnail. By pushing the abdominal wall outward with the thumb, a bulge was formed and an assistant could mark the point on the skin over the area using showeard paint.

3) *Observations and Discussion.*—The dermatome of the thirteenth thoracic spinal nerve was mapped on 3 animals. Two of the animals were cows and the other was a heifer calf. Photographs of 1 animal are shown in figures 3 and 4. The anterior border of this dermatome was posterior to the thirteenth thoracic vertebra. The area supplied by this nerve ran in a ventroposterior direction. It crossed the distal border of the thirteenth rib and the costochondral junction of the twelfth rib and ended posterior to the umbilicus. The posterior border of the dermatome of the thirteenth thoracic spinal nerve followed a course parallel to the anterior border of the dermatome. It did not include the anterior part of of the mammary gland. On 1 cow, there was an irregular enlargement of the area innervated anterior to the fold of the flank. The average area and the extreme limits of the area innervated by the thirteenth thoracic spinal nerve are shown in figure 5 (1a and 1b).

The dermatome of the first lumbar spinal nerve was mapped on 3 cows (fig. 5, 2a and 2b). Its anterior border began posterior to the first lumbar vertebra and, as it extended ventrally, passed in a ventroposterior direction. The anterior border included the costochondral junction of the thirteenth rib on 2 of the 3 cows. The posterior border included the skin on the anterior part of the mammary gland.

The dermatome of the second lumbar spinal nerve was mapped on 3 bulls and 1 heifer. Two of the bulls were yearlings and the other was an adult. The anterior border of the dermatome extended in a ventral and slightly posterior direction. The posterior border included the anterior edge of the base of the scrotum in the bulls and the anterior edge of the udder in the heifer. The average and extreme limits innervated by the dermatome in bulls are shown in figure 5 (4a and 4b) and the area innervated in the heifer is shown in figure 5 (3a and 3b).

The dermatome of the third lumbar spinal nerve was mapped on 2 bulls and 2 heifers. The dermatome included the skin over the tuber coxae, the lateral side of the thigh, and the scrotum on the bulls (fig. 5, 5a and 5b). The dermatome included all but the anterior edge of the udder of the heifers (fig. 5, 6a and 6b). The innervated area in the inguinal region extended farther posteriorly in the bulls than in the heifers.

On cow 26 (fig. 3 and 4), the anterior

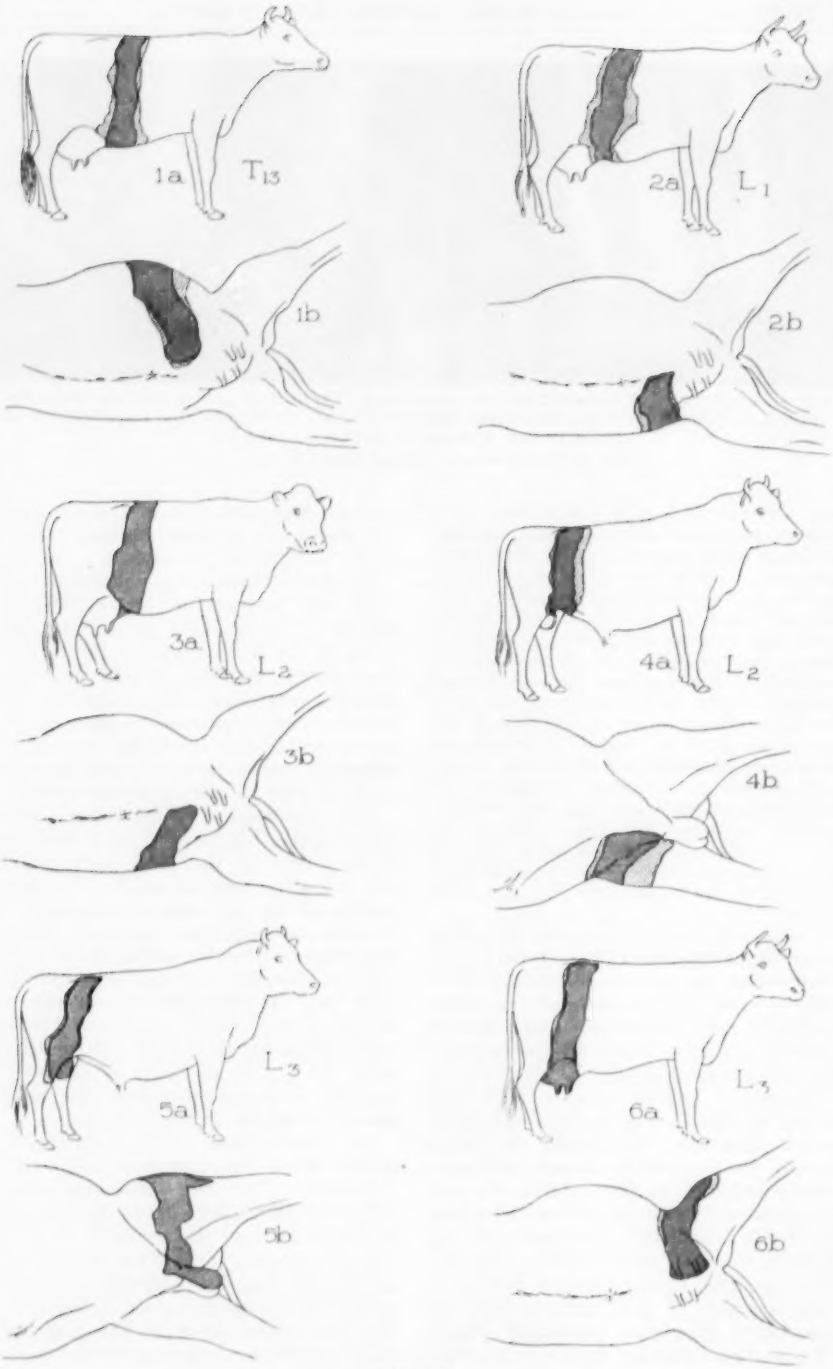

Figure 5

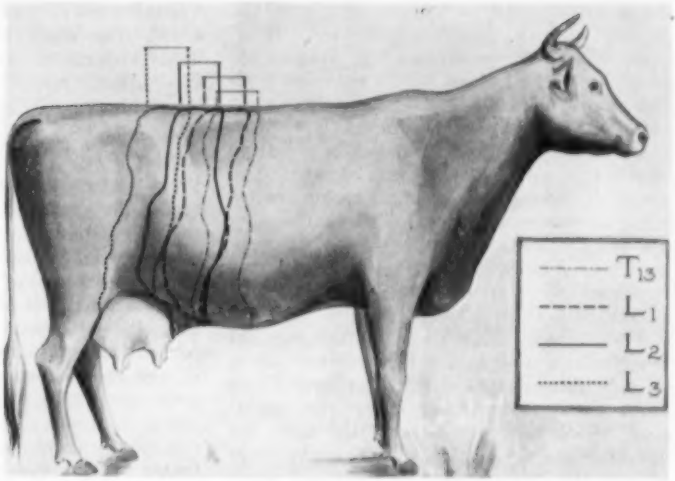

Fig. 6—Dermatomes projected on 1 animal to show overlapping.

limit of the area innervated by the fourth lumbar spinal nerve included the posterior half of the anterior teat. On another cow, the anterior border of the area supplied by the fifth lumbar spinal nerve included the posterior half of the posterior teat. The line of demarcation between sensitive and nonsensitive areas was sharp. The cows would kick vigorously when the anterior part of the respective teats was grasped with a pair of towel forceps. They would show no distress when the posterior part was grasped. However, if the apex of the teat were moved only slightly during this manipulation, they would kick vigorously.

The results of this study show that there is considerable variation among the different animals in the area innervated by a particular spinal nerve. The composite drawing (fig. 6), where the dermatomes for the thirteenth thoracic, the first, second, and third lumbar spinal nerves have been transposed on 1 animal, demonstrates that there is considerable overlapping of the various dermatomes. A small area of the lateral abdominal wall is innervated commonly by the last thoracic and the first and second lumbar spinal nerves.

It is apparent from this study that blocking only one of these spinal nerves will not result in desensitization of any skin area of the lateral abdominal wall. This is in agreement with Farquharson's observations. It appears that in order to achieve satisfactory anesthesia of the surgically important area of the lateral abdominal wall it would be necessary to block at least three spinal nerves.

The area of remaining sensitivity of the peritoneum was mapped in 2 cows and 2 heifers. These animals had been used to determine a dermatome by Sherrington's method of remaining sensitivity. All of the parietal peritoneum tested by scratching was sensitive. There was great variation in the degree of sensitivity as displayed by the actions of the animal. When the less sensitive areas were stimulated,

Legend for Illustrations on Opposite Page

Fig. 5—The lined areas represent the average extent of the dermatome on the experimental animals. The stippled areas represent the extreme limits of the dermatome on the experimental animals.

1a and 1b—Two views of the dermatome of the thirteenth thoracic spinal nerve.

2a and 2b—Two views of the dermatome of the first lumbar spinal nerve.

3a and 3b—Two views of the dermatome of the second lumbar spinal nerve on females.

4a and 4b—Two views of the dermatome of the second lumbar spinal nerve on males.

5a and 5b—Two views of the dermatome of the third lumbar spinal nerve on males.

6a and 6b—Two views of the dermatome of the third lumbar spinal nerve on females.

the animal would twitch the skin over the area or shift the position of its feet. However, when the more sensitive areas were stimulated, the animal would shift its position more vigorously and usually would kick on the stimulated side of the abdomen. The transition from a less sensitive to a more sensitive area was abrupt. The areas of greater sensation in the peritoneum corresponded so closely to the dermatomes mapped on the skin that they were not photographed. The areas of lesser sensitivity corresponded to the desensitized areas of the skin.

The presence of sensation in the parietal peritoneum under the desensitized areas of the skin suggests that sensory fibers from these areas must enter the spinal cord by other routes than through the dorsal rootlets of the spinal nerve supplying the dermatome. A possible route for these fibers is for them to originate in the dorsal root ganglia of the intact spinal nerve, pass to the sympathetic trunk via rami communicans and proceed anteriorly and posteriorly in the sympathetic trunk, then back to the adjacent spinal nerve by way of the rami communicans. Since the rami join the spinal nerves peripherally, these fibers would not be cut when the dorsal rootlets were severed. The results of this study do not prove or disprove this hypothesis. Other workers have presented more direct evidence in support of it. Van Harreveld and Smith [13] mapped dermatomes on the cat using Sherrington's method and then performed a sympathectomy. This resulted in the loss of pain sensitivity in a small area usually on the anteroventral side of the dermatome. They concluded that afferent fibers from the skin or skin vessels travel via the sympathetic chain. Pitkin [25] pointed out that in using segmental block for visceral surgery, the sympathetic nerves supplying that organ must also be blocked if the surgery is to be painless. Kuntz and Farnsworth,[18] using degeneration studies, concluded that afferent fibers travel in the sympathetic chain.

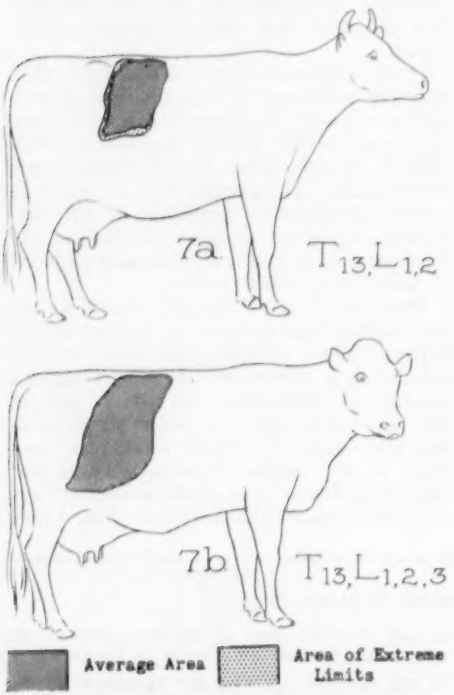

Fig. 7a—Area desensitized when the dorsolateral branches of T13, L1, and L2 were cut.

Fig. 7b—Area desensitized when the dorsolateral branches of T13, L1, L2, and L3 were cut.

B) Experimental Determination of the Peripheral Distribution of the Dorsolateral Branches of Spinal Nerves T13, L1, L2, and L3

1) *Review of Literature.*—Some of the inconsistent results associated with the use of paravertebral anesthesia have been explained by stating that only the dorsolateral branches of the spinal nerve had been blocked. As the distribution of the cutaneous area supplied by these nerves has not been determined, it was decided to include this in our experimental studies. In presenting the results of gross dissection, St. Clair [29] stated that the dorsolateral branches descend not lower than the fold of the flank. Similarly, Peterson [24] reported that the dorsolateral branches supply the skin of the upper flank region.

2) *Method of Procedure.*—Calves were used for this part of the study. The preoperative preparation was the same as for the dermatome studies. The skin was incised about 1 inch lateral to the midline on the side to be operated. The lumbodorsal fascia was incised and the multifidus dorsi and the longissimus dorsi muscles were divided by blunt dissection. The dorsolateral branches of the spinal nerves which were to be transected were located and followed proximally to near the intervertebral foramen. They were ligated with black silk, to mark them, and transected. The incision was closed with interrupted mattress and simple continuous sutures of silk. When the animal had recovered sufficiently from the surgery, the sensi-

tive and nonsensitive areas of the skin were mapped using the procedures described above for mapping the dermatomes. The animals were then photographed. At the time of necropsy, a segment of the dorsolateral branch of each of the spinal nerves transected was collected distal to the point of transection. These segments were fixed in a 10 per cent formalin solution. The segments were cut in transverse sections 5 μ thick for microscopic study. A section of each nerve was stained using a modified Holmes' technique[6] and another section with hematoxylin-eosin. The sections were studied microscopically for signs of degeneration.

3) *Observations and Discussion.*—The dorsolateral branches of the last thoracic and the first two lumbar spinal nerves were transected near the intervertebral foramen in 3 animals. Sections taken at the time of necropsy from the dorsolateral branches distal to the point of transection showed that all the fibers in these nerves were undergoing degeneration. Sections taken from similar points of the dorsolateral branches of the twelfth thoracic and the third lumbar spinal nerve showed no signs of degeneration on microscopic examination. The area desensitized when the dorsolateral branches of the last thoracic and the first two lumbar spinal nerves were transected was mapped on 3 animals and 1 of these animals was photographed (fig. 8). A composite drawing of the area desensitized in the 3 animals is shown (fig. 7a). The area extended from near the dorsal midline in a ventral and slightly posterior direction to about the middle of the lateral abdominal wall. The anterior border began posterior to the last rib and, as the border passed ventrally, it included the posterior border of the rib. The posterior border of nonsensitive area was anterior to the tuber coxae.

In 1 animal, the dorsolateral branch of the third lumbar spinal nerve was cut in addition to the last thoracic and the first two lumbar branches. The area desensitized by cutting these nerves was mapped and photographed (fig. 7b). The cutting of the dorsolateral branch of the third lumbar spinal nerve resulted in the area desensitized being enlarged in both posterior and ventral directions. The area extended to the anterior edge of the tuber coxae and ventrally to just above the fold of the flank. In all instances, the dorsolateral branches showed complete degeneration of the nerve fibers.

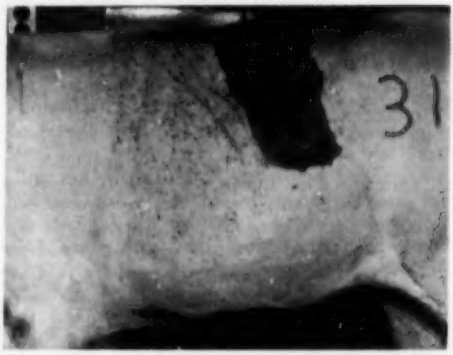

Fig. 8—Area desensitized when the dorsolateral branches of T13, L1, and L2 were cut.

SUMMARY AND CONCLUSIONS

The courses of the thirteenth thoracic and the lumbar spinal nerves were followed by dissection in 22 cattle. It was found that the spinal nerves follow a variable course in regard to the transverse process of their respective vertebrae. If the anesthetic solution is injected according to the landmark alone and not by finding the landmark and then locating the nerve, the solution may be deposited some distance from the nerve. This distance may be greater than the distance an anesthetic solution will diffuse through tissues.

The dorsolateral branch of the last thoracic and the lumbar spinal nerves passes above the intertransversales muscle and its strong fascia. This fascia is heavy enough to prevent an anesthetic solution from diffusing through it. This means that some of the anesthetic solution must be deposited above the fascia to anesthetize the dorsolateral branch of the spinal nerve and some must be deposited below the intertransversales fascia to anesthetize the ventral branch of the spinal nerve.

The dermatomes of the thirteenth thoracic and the first, second, and third lumbar spinal nerves were determined by Sherrington's method of remaining sensitivity. Twenty-two cattle were studied by this method. It was found that as many as three spinal nerves may innervate a given area of the skin of cattle. Thus, if only two nerves are blocked, the anesthesia would be unsatisfactory.

The area desensitized when the dorsolateral branches of the thirteenth thoracic and the first two lumbar spinal nerves

were cut was mapped on 3 animals. The area desensitized reached to about the middle of the lateral abdominal wall passing from the dorsal midline in a ventral direction. This indicates that, if satisfactory anesthesia is obtained in the upper part of the abdominal wall but the anesthesia does not extend below the middle of the wall, the ventral branches of the spinal nerves have not been blocked.

The sensitivity of the peritoneum was determined in 4 animals on which a dermatome had been mapped. The area of normal sensitivity corresponded quite closely to the isolated dermatome. However, there was some response to scratching the peritoneum under the nonsensitive skin.

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Histopathological Alterations of the Bovine Uterus
II. Uterine Tissue from Cows of Low Fertility

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A NUMBER of factors are involved in the greater recognition of bovine infertility. Dairy cattle have been selected primarily on the basis of milk production and body type with a rather general lack of consideration for other factors. The widespread usage of artificial insemination with its required record keeping has revealed that great numbers of otherwise apparently normal cows require repeated service prior to conception and that some never conceive. It was found by Tanabe and Casida [1] that approximately 50 per cent of all artificial inseminations were unsuccessful, thus indicating the magnitude of the problem.

Asdell [2] estimated that failures of reproduction among cattle in the State of New York accounted for a loss of 20 million dollars annually. In 1950, the U.S. Bureau of Animal Industry [3] estimated the total value of New York cattle at 387 million dollars and of all cattle in the United States at nearly 10 billion dollars. Thus, total losses from reproductive failures in cattle of the United States may be estimated at 500 million dollars annually.

The problem of low fertility in dairy cattle has been investigated from several aspects. Various attempts to incriminate bacteria, endocrine imbalances, management, and genetic factors have been described in the literature.

This study was undertaken in an effort to detect and evaluate, both macroscopically and microscopically, any tissue or cellular alteration which might provide a clue to the cause and nature of the syndrome associated with lowered fertility.

REVIEW OF LITERATURE

The various bacteriological studies of the bovine reproductive tract have been generally in agreement. Clark and Stevenson [4] studied the bacterial flora of the normal nongravid and gravid bovine uterus and found that 21 of 22 uteri in the study were free of bacteria. The exception, a nongravid uterus, yielded an aerobic gram-positive rod which was believed to be a contaminant. The survey conducted by Hatch *et al.*[5] on the reproductive tracts of infertile cows excluded known etiological agents, such as Brucella, Trichomonas, and Vibrio. Preliminary bacteriological study of the cervices and vaginas of infertile dairy cows indicated to Hatch and co-workers that bacterial infection, whether clinically apparent or not, may be responsible for many cases of infertility.

Weitz [10] examined the flora of the vaginas of 100 apparently normal dairy cows and concluded that *Corynebacterium renale* is a frequent and possibly normal resident of the posterior urogenital tract of dairy cows. *Corynebacterium pyogenes* was found to be associated frequently with an endometritis in cows characterized by a copious purulent discharge. Rowson [14] examined 296 female cattle that came to slaughter and found that 40, or 13.3 per cent, had ovarian bursal adhesions of varying degrees. Spriggs [15] examined 1,250 infertile cows and recognized clinical ovarian adhesions in 50 animals, 4.0 per cent of the total. The history of the group revealed that retained fetal membranes, pyoid discharge, ovarian cysts, tuberculosis, and rough rectal manipulation were all possibly implicated as contributory to the formation of adhesions. However, in 15 cases, an apparent cause could not be ascertained. Beaver *et al.*[16] studied 19 cases of sterility in cattle and stated that the theory of infection does not explain all cases. Hallman [17] followed the inflammatory and repair processes in uteri after injection of Lugol's solution. Woelffer [18] believed he obtained material enhancement of the conception rate of "shy breeding" cows by uterine infusion of either 3.5 per cent Lugol's solution or an aqueous solution of tyrothricin. Chambers [19] used a single inoculation of penicillin in the treatment of nonbreeding cows in dosages of 100,000 to 500,000 units in 10 to 50 ml. of sterile water. On the basis of field observations, he claimed that a regimen of treatment with penicillin at estrus and insemination at the ensuing period was 90.0 per cent effective in correcting infertility. Using uterine infusions of Ringer's solution, or solutions of iodine, tyrothricin, or penicillin and streptomycin, with or without sulfonamides, respectively, Roberts [20] was unable to detect beneficial results in repeat-breeding cows.

Asdell *et al.*[4] determined the model length of the estrous cycle in heifers and cows and empha-

Published with the approval of the director of the Wisconsin Agricultural Experiment Station as paper N8 218 from the Department of Veterinary Science, University of Wisconsin, Madison.

From a thesis submitted by Dr. Simon in partial fulfillment of the requirements for the Ph.D. degree at the University of Wisconsin, 1951. Supported in part by Badger Breeders Cooperative, Shawano, Wis.

sized the importance of the individuality in cycle lengths. Venzke [6] indicated that there is a definite relationship between disturbance of the endocrine system and lowered fertility and that proper controls for any use of an endocrine product be established, so that a critical evaluation could be accomplished. Hart [7] emphasized that considerable variation in chemical structure and physiological response exists with many available commercial hormone products.

Laing's observations [11] on the effect of management at mating upon bovine sterility showed that a higher rate of conception occurred as a result of free service. He attributed this to the closer relationship of service to the end of heat and, thus, to ovulation. His observations [12] on the survival period of the spermatozoa in the genital tract of the cow showed a direct relationship to fertility—the longer the survival period, the greater the fertility. In additional studies, Laing [13] was unable to correlate the percentage of abnormal spermatozoa ejaculated with the "service rating."

Gregory *et al.*[5] presented evidence to incriminate genetic factors in female sterility in dairy cows.

TABLE 1—Observations of Uteri of "Repeat Breeder" Cows

Cow	Cystic glands	Plasma cells	Lymph follicles	Eosinophils
COWS KILLED 3 DAYS AFTER SERVICE				
GROUP 1. NOVEMBER, 1947, TO APRIL, 1948				
G23	+	....	....	....
H45	+	....	....	....
H44 *	+	....	....	....
H2	....	+	....	....
H4 *	....	+	+	....
H8 *	....	+	....	....
H23	....	+	....	+
H5	....	+	....	....
H12	....	+	....	....
H33	....	+	+	+
H57	....	+	....	....
G2	....	....	....	+
G30	....	....	....	+
G47 *	....	....	....	+
H41	....	....	....	+
H58	....	....	....	+
H34	....	....	....	+
GROUP 2. OCTOBER, 1948, TO FEBRUARY, 1949				
G71	+	....	....	....
G5	....	+	....	....
G22	....	+	+	....
H50	....	+	....	....
G33	....	....	....	+
H46	....	....	....	+
H20	....	....	....	+
H87	....	....	....	+
COWS KILLED 20 TO 35 DAYS AFTER SERVICE				
GROUP 1. NOVEMBER, 1947, TO APRIL, 1948				
G27	+	....	....	....
H39	....	....	....	+
GROUP 2. OCTOBER, 1948, TO FEBRUARY, 1949				
G57	+	....	....	....
G58	+	....	+	....
G91	+	....	....	....
G97	+	....	+	....
G76	+	....	....	....
G100	+	....	....	....
G35	....	+	+	....
H61	....	+	....	....
G62	....	+	....	....
G64	....	....	+	....
G31	....	....	+	+
G38 *	....	....	....	+
G39	....	....	....	+

* Also showed tubal changes (*see* table 2).

MATERIALS AND METHODS

The tissues utilized in this study were collected from over 200 repeat-breeding cows, about equally divided between Holstein-Friesians and Guernseys, assembled from the northeastern Wisconsin counties served by the Badger Breeders Cooperative, Shawano, Wis.

TABLE 2—Oviduct Alterations According to Lombard [21]

Cow No.	Hydrosalpinx	Chronic interstitial salpingitis	Pyosalpinx
G46	+	....	....
H85	+	....	....
G43	+	....	....
H4 *	+	....	....
H8 *	+	....	....
H7	++	....	....
G93	....	++	....
H77	....	+	....
G36	....	++	....
H44 *	....	+	....
G47 *	....	....	++
H13	....	....	+
H40	....	....	+
G10	....	....	++
G38*	....	++	....
H94	....	++	....

+ = unilateral; ++ = bilateral.
* Also showed uterine changes (*see* table 1).

The cows were selected by employees of the Cooperative on the basis proposed by Tanabe and Casida.[1] That is, all animals had been bred at least four times and had failed to conceive, had dropped at least 1 normal calf previously, were less than 10 years old, and were apparently normal in every way except that they did not conceive. Not more than 2 cows were taken from any one herd.

The cows were divided into two groups scheduled to be slaughtered either on the third day or the twentieth to the thirty-fifth day after an experimental breeding. The latter were usually killed on the thirty-fourth day.

Tissue for sectioning was taken from each uterine horn and the uterine body and fixed in the following solutions: calcium cadmium formol, Mossman's, Helly's, and mercuric bichloride formol. Paraffin sections were cut transversely at 4 to 7 μ and stained with hematoxylin-eosin Y and Mallory's modification of Shorr's technique.

Studies were made of tissues of 87 repeat-breeding cows killed three days after an experimental breeding. They included 58 for the period of November, 1947, to April, 1948 (group 1), and 29 from October, 1948, to February, 1949 (group 2). Studies were made also of tissues from 22 repeat-breeding cows killed 20 to 35 days after experimental breeding. The study, therefore, actu-

ally comprised 109 of the 200 repeat-breeding cows.

Sections of the uteri were examined microscopically for the presence or absence in the endometrium of the following: glandular cysts, glandular exudate, lymph follicles, plasma cells, eosinophils, lymphocytes, polymorphonuclear leukocytes, mast cells, and subepithelial hemorrhage. These various elements were evaluated and compared with material from normal virgin heifers in an endeavor to determine whether any cellular characteristics might predominate.[20]

Results and Discussion

With the exception of cow H4, alterations within the endometria of the 109 cows were not of sufficient magnitude or character to account for the repeat breeding within any degree of certainty. Cow H4 had a definite endometritis as evidenced by a marked lymphocytic and plasma cell exudation. Numerous lymph follicles within the endometrium of this cow also attested to the presence of endometritis, past or present. In addition, the cow had unilateral hydrosalpinx.

The uteri of cows which contained cystic glands and what was thought to be more than normal numbers of lymph follicles, plasma cells, and eosinophils were tabulated (table 1). Since the significance of subepithelial hemorrhage and the presence of mast cells is unknown, these items were not included. In only 40 cows were any abnormalities detected. In nearly all cases, these were minimal or borderline. For example, only 11 of the cows had dilated or cystic uterine glands and, in some of these, only one or two cystic glands were found (fig. 1). In no case were more than 20 per cent of the glands cystic. Nine of the 11 cows had no evidence of endometritis. Increased numbers of lymph follicles

Fig. 1—Cross section of uterine glands, some of which are cystic and others contain cellular exudations within their lumens. x 165.

were found in the endometria of the remaining 2 (fig. 2).

Only 8 of the 40 cows had what might have been more than one abnormality. Included among these 8 is cow H4, the only frank case of endometritis.

As compared to virgin heifers, 14 of the cows had moderately increased numbers of lymph follicles. In about 5 per cent of the apparently normal virgin heifers, a limited number of lymph follicles were detected within the endometria. Such lymph follicles may increase in numbers in endometritis, perhaps only in certain types, but how long they persist after recovery is not known.

Among control animals, virgin heifers, an occasional individual was found where the endometrium was rather heavily infiltrated with eosinophils. It is not surprising that 16 of the 109 repeat-breeding cows showed a like condition but it is unlikely that this interfered with reproduction. Nothing significant was found regarding the number and distribution of polymorphonuclear leukocytes (heterophils) within the endometria of any of these cows. This might be difficult to evaluate in the cows slaughtered three days after breeding, because Weber [20] showed that on the second day postestrus there was an enormous infiltration of these cells into the endometrium and a like outpouring of them into the uterine lumen in the normal estrous cycle. By the third day, this was considerably cleared.

Lombard [21] studied the oviducts of 154 repeat-breeding cows including essentially all of the 109 cows reported here. She found alterations within the oviducts that would account for the repeat breeding either in part or entirely in 16 of the 154. The other 138 were without tubal alterations. The changes within the abnormal oviducts are listed (table 2). If cow H4 is not considered, it will be seen that only 4 cows with tubal alterations, H8, H44, G47, and G38, are listed in tables 1 and 2,

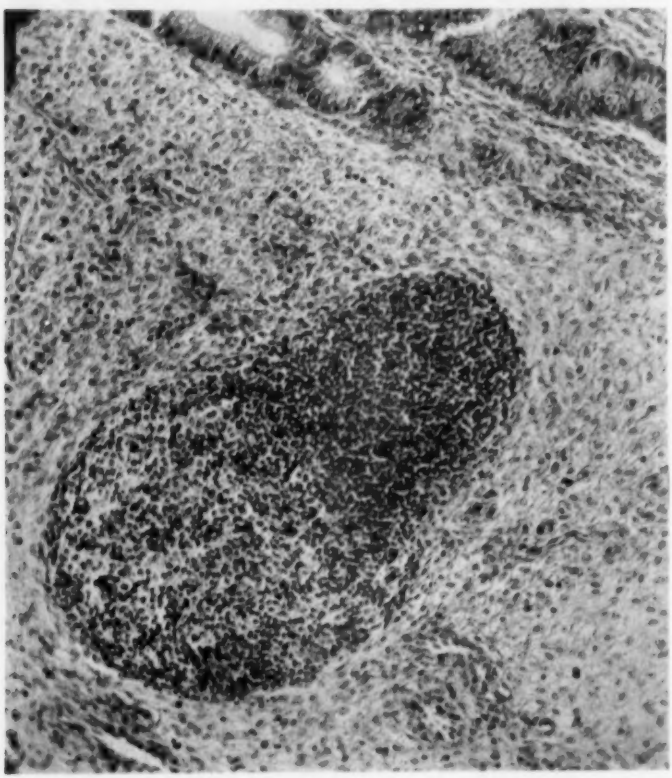

Fig. 2—Cross section of a large lymph follicle in the endometrium of cow H4. x 165.

cows in which conditions were found that were suggestive of abnormalities. It is readily seen that within this number of cows there was no correlation between the known tubal alterations and the suspected endometrial alterations, a situation that casts further doubt on the proposal that suspected endometrial alterations are indeed significantly pathological. All of these suspected abnormal changes might be within the normal physiological limits of the bovine uterus, again excepting cow H4.

That the slight evidence of endometritis found in the 39 cows is of minor significance is strengthened by the negative bacteriological examinations of the uteri. It is possible that an acute temporary endometritis could have been a contributory factor to repeat breeding in these cows but the probability that such an endometritis might develop at relatively frequent intervals is remote. Finally, the factors responsible for not more than 15 per cent of the repeat breeding were accounted for by pathological changes in the oviducts and uterus. More than this, the study did not provide definite clues as to the mechanisms of "embryonic death" which was shown by Tanabe and Casida [1] to be a most significant factor in lowered fertility.

SUMMARY

1) The endometria of repeat-breeding cows did not differ greatly from that of apparently normal virgin heifers; in most cases, no true differences could be detected.

2) Endometritis was not a major cause of repeat breeding in otherwise apparently normal repeat-breeding cows.

3) The study did not provide positive clues as to the mechanism of embryonic death.

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Infectious Bovine Rhinotracheitis—A Newly Recognized Virus Disease of Cattle

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In 1953, Ramsey and Chivers[1] described an apparently new disease of cattle in Iowa, characterized by severe diarrhea and the development of erosions on the mucous membranes of portions of the digestive tract. Because of this latter feature, they named the condition "mucosal disease." A somewhat similar syndrome affecting cattle in Indiana was later described by Pritchard *et al.*[2] Though resembling mucosal disease in many respects, it more closely resembled virus diarrhea (VD), originally described by Olafson *et al.*[3] in New York State in 1946. The newly discovered disease in Indiana, therefore, became known as "virus diarrhea-Indiana."

At approximately the time virus diarrhea-Indiana was first observed, a condition appeared among dairy cattle in Los Angeles County[4,5] that bore certain resemblances to mucosal disease, the chief being a marked congestion of the mucosa of the upper respiratory tract, and sometimes of the esophagus. The disease became known as an "acute upper respiratory infection"[4] and as an "influenza-like disease" of cattle.[6] Later, a clinically similar although more severe condition appeared in feedlot cattle in several areas in California[6] and was referred to as simply a "respiratory disease" of cattle. Meanwhile, Colorado investigators described a respiratory disease that had been prevalent in feeder cattle in Colorado since 1950.[7] This condition was designated as "infectious necrotic rhinotracheitis" by Miller,[7] and as "infectious rhinotracheitis" by Jensen *et al.*[8] Though characterized by a respiratory tract involvement somewhat similar to that reported from California, early descriptions of the disease indicated that it also shared characteristics in common with mucosal disease.

Because of the possible relationship of the diseases reported from California, Colorado, and Indiana to mucosal disease, and possibly to VD, all were grouped together and referred to as the "mucosal disease complex." Rinderpest was also included in this group.

Closer liaison between the investigators of these various diseases indicated that two distinct groups of diseases were involved—one primarily of the respiratory tract and the other of the digestive system. The entities of the respiratory disease group could be readily transmitted by intranasal exposure to nasal secretions of affected cattle.[9] It had been determined previously that members of the true mucosal complex were transmissible by means of blood and spleen given parenterally.[2,3,10] An effort was then made to determine the relationship between the members of the respiratory disease group, namely, the acute upper respiratory infection of California dairy cattle, the respiratory disease of California feeder cattle, and infectious rhinotracheitis of Colorado feeder cattle.

Cross-protection tests in calves established that the conditions reported in feeder cattle in California and Colorado were undoubtedly the same disease entity.[9] Since no description of such a disease could be found, it was considered a new disease and a recommendation was made that it be named "infectious bovine rhinotracheitis" (IBR).[9] The results of the preliminary tests were confirmed by recipro-

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The authors gratefully acknowledge the excellent cooperation received in the studies reported herein from many feedlot and dairy farm operators, and from veterinarians in private practice and in government service. They are particularly indebted to Dr. Rue Jensen, School of Veterinary Medicine, Colorado A. & M. College, Fort Collins, for making it possible to obtain materials from cases of rhinotracheitis in Colorado, and to Dr. H. E. Adler, School of Veterinary Medicine, University of California, Davis, for demonstrating by cultural studies that the agents isolated were not PPLO. Acknowledgments are due also to Mr. J. K. Saito, Miss E. M. Wada, and Mr. K. E. Conklin, of the School of Veterinary Medicine at Davis, for technical services rendered.

Financial assistance for this study was furnished, in part, by the Animal Disease and Parasite Research Branch, Agricultural Research Service, U.S. Department of Agriculture.

cal cross-protection tests in calves, which also established that the infection which had occurred in dairy cattle in Los Angeles was the same. Finally, the causative agent was isolated in tissue culture.[11] Using a culture of this isolate, reciprocal cross-neutralization and protection tests confirmed the results of the earlier tests.

Since it has not been done previously, IBR will be described in detail as a specific disease entity, and pertinent reports will be reviewed. To provide a complete survey in one publication, details of the unpublished findings referred to above will be included, as well as the results of studies by some of the present authors that have appeared only as preliminary publications.

Occurrence and Distribution

The origin of IBR is unknown. Whether is has existed for some time, as certain veterinarians and stockmen believe, and has only recently been detected because of increased severity and distribution or whether it is a recent development will probably never be ascertained. In the absence of documented evidence, it may be assumed that it has appeared in its present form only recently. The possibility of its being related to the shipping fever-pneumonia complex of cattle awaits further clarification but indications of a relationship are slight.

While the presence of the infection has been confirmed experimentally in California and Colorado only, clinical reports indicate a much wider distribution (Wyoming, Montana, Arizona, Illinois, and Idaho).[12]

To date, IBR has been reported only where the concentration of cattle is high, as in large feedlots and dairy operations. It has not been reported in range cattle.

Information is meager on the breed susceptibility to the naturally occurring disease, since practically all of the feeder cattle involved thus far have been Herefords and the dairy animals have been almost exclusively Holstein-Friesians. Experimentally, calves of the above breeds and Jerseys and Guernseys, irrespective of sex, were found equally susceptible. Likewise, it is difficult to determine the susceptible age range of cattle since all or most epizootics, at least in California, have occurred on premises where stock were over 6 months of age. Experimentally, however, calves as young as 6 weeks old regularly develop as severe a form of the disease as do older animals.

Epizootiology

Since the etiological agent has only recently been isolated, little opportunity has been afforded to study the epizootiology of IBR.

It is apparently not seasonal; of late, it has been prevalent in feeder cattle throughout the year. In California, in both dairy and feeder cattle, it has been most severe during the fall months. It generally occurs after the introduction of new cattle. Feeder cattle in which this sequence has been consistently observed have frequently passed through several feedyards, in state as well as out of state. Its occurrence in dairy cattle has sometimes coincided with the importation of replacement stock.

The disease appears to fall into two categories—a mild type predominating in dairy cattle and a severe type in beef cattle. The pattern differs somewhat between dairy and beef breeds. The dairy cattle type tends to appear almost simultaneously in one or more herds which are close to each other but not necessarily adjacent. Or it may appear at some distance from the nearest active known infection. In general, the disease tends to progress through an area until most of the herds become involved. Its duration and severity vary considerably between herds as well as individual animals. Mortality is extremely low.

In feedlots, one or more cases usually appear simultaneously in each of several pens scattered throughout the yard, suggesting that the disease arises from a number of foci rather than from a central focus. Additional pens become involved on consecutive days until all or most eventually contain infected animals. The maximum number of new cases, on a daily basis, usually occurs during the second and third week, with the disease running its course by the end of the fourth to the sixth week; occasionally, it has persisted in a feedlot for several months. Apparently, the greater the number of animals affected, the more severe are the cases and the longer the disease persists.

Susceptible cattle brought to a feedlot where the disease is in progress or has only

recently subsided usually develop the infection ten days to three weeks later. Little or no similar information is available on dairy cattle.

While the initial appearance of IBR in both dairy and beef herds frequently attains epizootic proportions, subsequent outbreaks, particularly in dairy cattle, are mainly sporadic and involve relatively few animals. Such cases are usually mild and are found in animals not previously affected. In feedlots, where susceptible animals are constantly being introduced, subsequent outbreaks are usually severe and, as in dairy herds, previously affected animals are apparently immune.

Signs and Courses

The onset of the disease is sudden. In dairy cattle, an abrupt cessation of milk flow is the first indication of illness but, in beef cattle, the corresponding early signs are so mild that they may be detected only by those who have had considerable clinical experience with the condition. Salivation, a slight serous nasal discharge, congestion of the nasal mucosa, a temperature range from 104 to 108 F. or higher, and often a manifestation of a certain amount of hyperexcitability are the earliest definite observable signs. The extent of the febrile reaction is out of proportion to the alert appearance of the affected cattle (fig. 1). Respirations are accelerated and shallow, and a degree of dyspnea is apparent, particularly if the cattle are moved or hurried. However, the lung sounds are essentially normal. Leukopenia has not been detected at any stage of the infection.

Fig. 1—Animal with an early acute case of infectious bovine rhinotracheitis showing slight nasal discharge. Note the alert appearance of the animal.

Fig. 2—Animal with an advanced case of infectious bovine rhinotracheitis showing respiratory distress.

In mild cases, which occur more frequently in dairy than in feeder cattle, the temperature drops rapidly after 24 to 48 hours to within 1 or 2 degrees of normal, salivation and nasal discharge diminish, and the animals usually recover completely within ten days to two weeks. In such cases, little loss of condition is suffered and milking cows quickly return to full production. (Occassionally, deaths occur during the acute febrile stage of the infection, particularly in feeder cattle.) In more severely affected animals, the temperature remains elevated longer, the nasal discharge becomes progressively more copious and purulent, and there is a marked congestion of the nasal mucosa. The appetite diminishes but inappetence is rarely complete at this stage. After several days, the temperature subsides to within 2 or 3 degrees of normal, where it remains until the animals either show improvement or die.

Animals in the advanced stages of the disease experience respiratory distress, exhibit open-mouth breathing, become completely inappetent, and lose weight rapidly (fig. 2). In the terminal stages, they assume either a stiff, stilted stance or remain recumbent much of the time (fig. 3).

They usually die, due to a secondary bronchopneumonia. Those that recover require several months to regain their former condition; an occasional animal never recovers completely.

An unusual feature of the disease, observed infrequently in dairy cattle, is the development of a marked emphysema posterior to the withers, along the belly, over the gluteal region, and along the posterior aspects of the thighs. In several instances, in California, some affected animals developed a lameness in the rear limbs that occasionally progressed to a complete posterior paralysis. However, the inconstancy of this finding suggested that it was a coincidental occurrence rather than one related to the disease. Occasionally, experimentally infected calves, as well as in field cases, developed a conjunctivitis, but this is also regarded as unrelated to the IBR infection. In Colorado, the infection is often accompanied by diarrhea and a considerable percentage of the pregnant animals abort. Abortions have not been reported in California but the number of pregnant animals involved has been negligible.

Morbidity and Mortality

In the original report of IBR in Los Angeles County,[4] which involved 52 dairy herds comprising more than 13,000 animals, it was indicated that 7.6 per cent of the animals became infected, and 3.0 per cent of the infected cattle died. Morbidity and mortality varied considerably with individual herds.

The morbidity and mortality figures are higher for beef cattle in feedlots but, as in the case of dairy cattle, they vary widely. A morbidity of 30 to 100 per cent was reported[7] in Colorado feeder cattle, with a herd mortality from 2 to 10 per cent. In an extensive and prolonged outbreak in a large feedlot in California, Hoadley[13] found that of 7,189 animals on the premises, 1,692 (22.1 %) became infected and 203 (12.0 %) of the infected animals died. These figures are believed to be representative of the disease as it occurs in California feedlots, and the morbidity and mortality when the disease recurs in feedlots appear to be slightly, if at all, lower than in the initial infection. This may be due to the high rate of turnover in feedlots as compared with dairy operations, with the result that susceptible animals are constantly being introduced.

Gross Pathology

The principal lesions are limited to the nasal cavity, larynx, pharynx, and trachea, ending abruptly in the major bronchi. In mild cases, no changes can be detected externally other than hyperemia of the muzzle and of the visible nasal mucosa. In both mild and severe cases, the mucosa of the entire nasal tract is congested, swollen, and contains petechiae. In mild cases, the exudate is slight, whereas in severe infections it is copious, fibrinopurulent, and loosely attached to the surface of the mucosa, partially occluding the meatuses (fig. 4). When the exudate is removed, the mucosal surface exposed is intact, reddened, and sometimes has necrotic foci. Jensen *et al.*[8] observed that the ventral meatus and ventral turbinates are more severely affected than the dorsum of the nasal cavity; also that the palatine and maxillary sinuses are partially filled with a mucopurulent exudate.

Fig. 3—Animal in terminal stages of infectious bovine rhinotracheitis infection.

In mild and early cases, the mucosa of the pharynx and larynx is hyperemic. Later it becomes covered with a mucopurulent, fibrinous exudate. In mild cases, relatively small amounts of catarrhal exudate are present in the trachea. The anterior tracheal mucosa of severely affected animals is deeply congested and covered by a layer of mucopurulent, fibrinous exudate of variable thickness. The underlying surface is usually denuded of epithelium. The lymph nodes involved are swollen, reddened, and edematous.

Apart from the occasional presence of pulmonary emphysema, the lungs are essentially normal, except in fatal field cases in which bronchopneumonia is almost always present. Miller [7] reported acute inflammation and frequently ulceration of the mucosa of the abomasum, severe enteritis in the small intestine, and a mild inflammation in the large intestine. These changes have not been observed, by the authors, in either field or experimental cases in California.

In experimentally produced IBR, the changes are essentially the same; however, the rhinitis is more severe and the tracheitis less severe than in the naturally occurring infections.

HISTOPATHOLOGY

There is nothing particularly characteristic about the histopathological changes in IBR. Mild cases show excessive mucus in the epithelium of the nasal cavity, larynx, and trachea; edema of the mucosa, with emigration of neutrophils through the epithelium; and mononuclear cells, consisting of lymphocytes, macrophages, and plasma cells infiltrating the submucosa. Abscesses and eruptions of solitary lymph follicles also occur.

In the respiratory tract of severely affected animals, a fibrinous pseudomembrane that is densely infiltrated with neutrophils covers the epithelium and denudes it at points of its attachment. Focal necrosis of the epiglottal mucosa with dense collections of mononuclears in the submucosa is sometimes observed. In most fatal field cases, the lung sections show changes typical of bronchopneumonia. Bronchi and bronchioles are filled with purulent exudate, and peribronchiolar alveoli show edema and neutrophil invasion. Lymph nodes draining inflamed areas exhibit diffuse lymphoid hyperplasia and hyperemic cortical foci. Inclusion bodies have not been observed in this disease.

Most cases of IBR are manifested as a mild inflammation of the upper respiratory tract; fibrinonecrotic exudate forms only in exceptional cases, following necrosis of the epithelium and invasion of the unprotected underlying tissue by bacteria. The more extensive lesions undoubtedly develop as a result of this invasion. The pure viral infection is probably no more than an acute catarrhal inflammation.

DIAGNOSIS

Infectious bovine rhinotracheitis can be diagnosed on the basis of history, the onset pattern, clinical signs, and the course

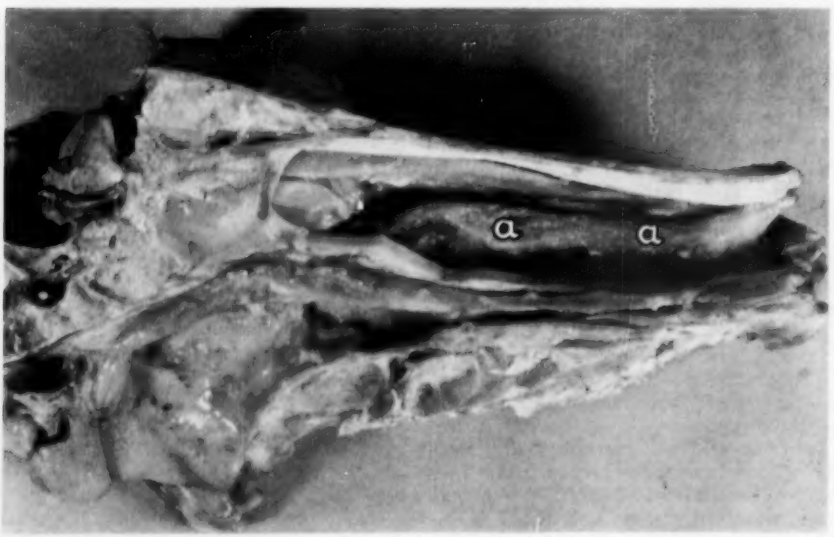


Fig. 4—Saggital section of nasal cavity and pharynx of calf showing pseudomembrane (a) consisting of fibrinonecrotic exudate.

of the disease, particularly in areas where it is known to exist. Necrotic laryngitis may be confused with IBR because of clinical similarity but, at necropsy, the two can be differentiated. The absence of lung involvement and failure to respond to antibiotics or chemotherapy distinguish IBR from primary pneumonia, although advanced cases of IBR are usually complicated by a secondary pneumonia. History is also of value in differentiating IBR from shipping fever. Moreover, the marked depression characteristic of shipping fever is absent in IBR, particularly in the early stages of the disease.

Infectious bovine rhinotracheitis can be specifically diagnosed by isolation and identification of the causative virus and by protection tests in cattle.

PREVENTION, TREATMENT, AND CONTROL

No specific immunizing agent is available for IBR. Limited attempts at prevention by the injection of convalescent cattle serum have given rather promising results in feeder cattle, and further studies along these lines are recommended. Anti-hemorrhagic septicemia serum has been used therapeutically, but with little or no effect.

Attempts have been made to treat IBR with various compounds.[14] In the early stages of the disease when the infection seems to be primarily viral, antibiotic therapy is apparently of no value; the virus is not sensitive to penicillin or streptomycin. However, since secondary bacterial invaders undoubtedly predominate during the later stages of the infection, routine treatment is recommended as a means of reducing losses due to these organisms. Several enzymic compounds that liquefy the exudate that collects in the respiratory passages have been used to relieve the nasal and tracheal congestion.[15]

The disease is seemingly not highly transmissible unless the animals are in close contact. If possible, therefore, sick cattle should be segregated as soon as the illness is detected. Young cattle brought to infected premises should be kept from contact with older animals which might harbor and disseminate the virus.

EXPERIMENTAL

Transmission Studies.—McIntyre [5] reported successful transmission to calves of the disease later known as IBR by inoculation of blood, nasal secretions, and feces. However, since combined inoculums and various routes were used, it was impossible to determine which of the inoculated materials contained the infectious agent, and which was the most effective route of inoculation. Moreover, since not all of the inoculums were bacteria-free, the nature of the transmitted agent could not be ascertained. However, since several of the experimentally infected calves developed a mild leukopenia with pyrexia, it was thought possible that the disease under study was VD.

McKercher *et al.*,[6] working in an area where the disease did not exist, made a number of unsuccessful attempts to transmit an infectious agent from specimens of blood, spleen, and lung obtained from diseased animals. It was definitely established, however, that the disease was not VD.[6]

When IBR later appeared among feeder cattle in California, renewed efforts were made [9] to reproduce the condition in yearling cattle and calves, using febrile blood as the inoculum. Again, the results were negative. However, nasal washings from animals in the early febrile stage of the naturally occurring disease, when introduced into the nasal tract with an ordinary fly sprayer, readily reproduced the infection in calves from 4 to 6 months old. Though the nasal washings frequently yielded Pasteurella on culture, washings that had been rendered bacteriologically sterile with antibiotics were equally infectious for calves. From this, it was inferred that the etiological agent was a virus. It was subsequently shown that the agent was present only in cattle affected with the disease, that adult cattle were as susceptible as calves to experimental infection, and that the experimental infection could be transmitted by pen contact.[9]

A finding of extreme significance was that the identical clinical syndrome could be produced in calves by inoculation of nasal washings from animals with either the California dairy cattle or Colorado feeder cattle disease. However, the report of the Colorado investigators [16] that rhinotracheitis could be transmitted by the inoculation of blood serum and tissues from animals with fatal infections prompted the California workers to attempt to duplicate this finding. Accordingly, calves with rhinotracheitis, produced by the in-

oculation of nasal washings from animals with field cases of the disease in Colorado, were bled at 48-hour postinoculation intervals from the second to the tenth day. The blood samples were defibrinated, fast frozen, and held under dry-ice refrigeration in sealed glass ampoules. Several weeks later, they were thawed, pooled, and inoculated, both intravenously and subcutaneously, into calves, the amounts given ranging from 10 to 20 ml. per animal. In one experiment, blood was sprayed into the nasal passages as for the inoculation of nasal washings. For three weeks after inoculation, temperatures were taken twice daily and the animals were examined daily. All the inoculated calves remained clinically normal and no unusual responses were recorded. Later, attempts to recover the virus from the lungs, liver, and spleen of experimentally infected calves were unsuccessful.[11]

TABLE 1—Cytopathogenic Changes Produced in Tissue Culture Cells by Inoculation of Nasal Washings from Animals with Respiratory Disease (California Beef Cattle) and Rhinotracheitis (Colorado Beef Cattle)

Inoculum	Tissue cultures of					
	BEK	SK	CEF	Hela	KB	L
Nasal washings, respiratory disease (Calif.)	+	—	—	—	—	—
Nasal washings, rhinotracheitis (Colo.)	+	—	—	—	—	—

BEK = beef embryo kidney; SK = swine kidney; CEF = chicken embryo fibroblasts; + = cytopathogenic effect; — = no cytopathogenic effect.

The results of these transmission studies established that the clinical syndrome under study was transmissible and that the infectious agent was confined to the upper respiratory tract.

Etiological Studies.—Numerous attempts were made to recover the etiological agent of IBR by injection of blood, spleen, lung, and nasal secretions, and on one occasion, milk, from animals in early and late stages of the disease, into guinea pigs, suckling and weaned mice, and embryonating hen eggs. Guinea pigs were inoculated by various parenteral routes, the mice were injected intranasally, intrathoracically, intraperitoneally, and intracerebrally, while the embryonating hen eggs were inoculated by all recognized routes and then incubated at various temperatures. Whenever original passage failed to result in the recovery of an agent, a variable number of blind passages were made for each specimen inoculated; the results were always negative. When it was definitely ascertained that the infectious agent was present only in the nasal secretions of infected animals, isolation attempts were concentrated on this one material. Again, however, results were negative until tissue culture was employed.

Nasal washings representing the fifth cattle passage of material from the respiratory disease in California beef cattle, and the third from animals with rhinotracheitis in Colorado, were used as inoculums for tissue cultures in attempts to propagate the etiological agent of each condition. Nasal washings from cattle not known to have been affected with either condition served as the control inoculum.

The three samples of nasal washings were diluted 1:10 in lactalbumin-tris (LT) medium fortified with an additional 1,000 units of penicillin and 1,000 μg. of streptomycin per milliliter. Cultures of beef embryo kidney (BEK), adult swine kidney (SK), and chicken embryo fibroblasts (CEF) were prepared by a modification of the method described by Youngner,[17] and Madin *et al.*,[18] using LT as the nutrient medium. Two tubes of the different tissue types were inoculated with 2 ml. of each of the three samples. HeLa, L, and KB cells were also inoculated, as described above, with each specimen. The tubes were incubated at 37 C. and examined 24 and 40 hours after inoculation for cytopathogenic (CP) changes.

After 40 hours' incubation the cells in the BEK cultures inoculated with the California and the Colorado materials exhibited CP effects in that they became rounded and granular and, shortly thereafter, separated from the glass surface. The tubes inoculated with the control nasal washings did not manifest these changes and failed to show any comparative CP effect, even with continued serial passage. The CP changes were readily produced in the BEK cultures on serial passage, the effect being evident 24 hours after the first passage, while at 48 hours it was complete with all the cells in suspension (table 1).

Before proceeding with pathogenicity studies of the isolates in cattle, it was thought advisable to eliminate the possibility that they might be pleuropneumonia-like organisms (PPLO), which are sometimes found in upper respiratory passages. Accordingly, mediums used to cultivate the most fastidious of the PPLO were inoculated with each isolate. The failure of growth was regarded as evidence that isolates were not PPLO. Furthermore, each agent could pass through a fine, sintered-glass filter. These findings definitely established that the isolates were viruses.

To determine whether the isolated agents were the cause of the disease in the cattle from which they were obtained, calves were inoculated intranasally, as previously described, using 2 animals for each culture. Each animal was inoculated with 15 to 20 ml. of undiluted, fourth passage tissue culture of each of the first two isolates. Uninoculated nutrient fluid, harvested from tissue cultures, was also inoculated into 2 additional calves. The test animals were separated to

avoid cross infection. Temperatures of the inoculated animals were recorded twice daily and white blood cell (w.b.c.) counts and physical examinations were made once daily.

The 4 calves that received either culture of the tissue culture-propagated agents developed a marked fever, which usually began within 24 hours and reached a peak on the third or fourth day after inoculation. Both control calves remained clinically normal with no unusual fluctuations in temperature. Clinically, the infection was identical in the two groups of calves and identical with that produced previously by inoculation of nasal washings from animals with the California or Colorado diseases. The w.b.c. counts fluctuated somewhat but remained well within the normal range.

Titration of the Virus Isolates.—Each isolate at the fourth, tissue culture-passage level was titrated in tubes of BEK. Eight tubes were used for each of the decimal dilutions, which ranged from 10^{-3} to 10^{-7}. Lactalbumin-tris medium was the diluent used and 0.2 ml. of each dilution was inoculated per tube. After three days' incubation at 37 C., readings were made and the titer calculated by the method of Reed and Muench.[19] The titers of both cultures were practically identical (table 2).

Since each isolate produced in calves a clinical syndrome identical to that produced by nasal washings of cattle affected with either the California beef cattle respiratory disease or rhinotracheitis, it was concluded that these agents were the etiological factors of the syndromes under study. The fact that both isolates behaved similarly in tissue culture and attained practically identical titers in this medium suggested that they might be the same virus.

Immological Studies.—Cross-Protection Tests.—Prior to the isolations made in tissue culture, it was found that calves that had recovered from experimental infection with the respiratory disease of California beef cattle were resistant to homologous challenge. Therefore, a preliminary trial was set up in which calves that had recovered from the beef cattle respiratory disease (California) and rhinotracheitis (Colorado) were challenged with nasal secretions from cases of rhinotracheitis (Colorado). The results[9] provided strong evidence that the syndromes that had occurred in California and Colorado beef cattle were the same disease entity. It was recommended that the disease be known as "infectious bovine rhinotracheitis" (IBR).

In a more comprehensive experiment, reciprocal cross-protection tests were conducted on isolated groups of calves, 5 to 9 months old, which had recovered from each

TABLE 2—Titration of California and Colorado Isolates in Cultures of Beef Embryo Kidney

Inoculum	Dilutions of inoculum					t.c.i.d.$_{50}$ titer
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
California isolate	8/8*	8/8	8/8	7/8	0/8	6.4
Colorado isolate	8/8	8/8	8/8	7/8	1/8	6.5

* Number of tubes showing cytopathogenic changes/number of tubes inoculated.

of the three syndromes, namely, the acute upper respiratory infection of California dairy cattle, rhinotracheitis of Colorado feedlot cattle, and the respiratory disease of California feedlot cattle. Their periods of recovery had varied from three weeks to six months.

The test animals were allotted to three groups, containing representative calves recovered from each of the three conditions under study, together with 1 previously unexposed calf of comparable age to serve as the challenge control. To minimize cross exposure after challenge, the groups were separated as widely as the facilities permitted. Two groups were placed at the opposite ends of the same building, and several normal calves were placed in an intervening pen to check the effectiveness of the security measures. The building had recently been thoroughly cleaned and disinfected. The third experimental group was placed in a small outside paddock that had not contained infected animals for several months previously. Coveralls and gum-rubber boots were placed in each of the experimental pens for use by the attendants while caring for and examining the animals; also their hands were thoroughly scrubbed between each group examination.

A sample of blood was obtained from at least 1 animal in each group, and all were then given the appropriate inoculum by nasal spray, consisting of 25 to 40 ml. of crude, untreated, nasal washings from animals with typical field and experimentally produced cases of each of the three conditions under study. The temperature of each animal was recorded twice daily, and

close clinical observations were made for 30 days following inoculation.

The 3 control animals developed the typical clinical infection on the third day after inoculation, with a temperature peak on the fourth day for 2 and on the fifth day for the third. The recovered test animals showed no evidence of clinical infection, apart from several insignificant febrile irregularities.

The febrile reaction of one of the 3 control animals is contrasted with that of 1 homologously challenged and with those of 2 cross-challenged animals (graph 1). The results of this experiment are summarized (table 3).

From these results, it was concluded that the California and Colorado beef cattle disease and the California dairy cattle disease were the same entity.

Shortly after this experiment ended, two isolations of a CP agent were made, one from animals with experimentally produced IBR of California origin, the other of Colorado origin. (Later, a culturally identical isolate was recovered from infected California dairy cattle.)

TABLE 3—Cross-Protection Studies in Calves

Challenge inoculum*	Recovered from experimental infection with			
	Rhinotracheitis (Colo.)	Respiratory disease (Calif.)		Controls
		Beef cattle origin	Dairy cattle origin	
Rhinotracheitis (Colo.)	0/3†	0/2	0/2	1/1
Beef cattle, respiratory disease (Calif.)	0/2	0/1	0/2	1/1
Dairy cattle, respiratory disease (Calif.)	0/2	0/2	0/1	1/1

* Nasal washings. † Number reacting/number inoculated.

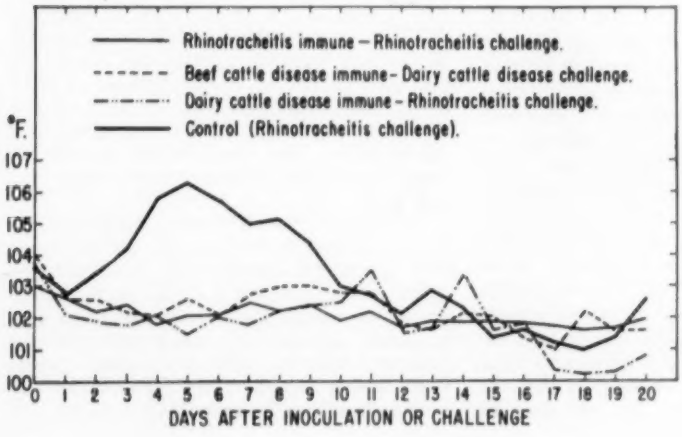
On inoculation into calves, each agent* produced the identical clinical syndrome as that in the animals from which it was isolated. After recovery, the calves were challenged with the homologous or the heterologous tissue culture-propagated isolate at the fifth passage level. Two control calves were also inoculated.

As in the previous experiment, the recovered calves were refractory to both the homologous and the heterologous challenge inoculations, whereas the control animals developed the characteristic infection in each case. Evidence was thus provided that the isolates were identical.

Serum-Virus Neutralization Tests in Tissue Culture.—Comparative serological studies of the California and the Colorado beef cattle isolates* were conducted to confirm their identity with each other and with the infectious agent found in the nasal secretions of animals with experimentally produced IBR. To demonstrate the latter relationship, paired samples of preinoculation and convalescent serums from calves that had been infected with nasal washings of animals with experimentally produced IBR were used, rather than serums of calves which had been infected with the tissue culture-propagated agents.

The serums were inactivated at 56 C. for 30 minutes, and dilutions of 1:2½, 1:5, 1:50, and

* The isolate from California dairy cattle was not studied in parallel with the other two at this time. Subsequently, however, it was shown to be identical with them.



Graph 1—Response of recovered calves to homologous and to heterologous challenge with nasal washings.

1:500 of each were then prepared in LT medium. To each dilution were added 1,000 t.c.i.d.$_{50}$ of either the California or the Colorado isolate in an equal volume of LT medium, giving final serum dilutions of 1:5, 1:10, 1:100, and 1:1,000. The mixtures were held at room temperature for one hour and then inoculated into tubes of BEK cultures, using eight tubes for each dilution of serum and virus. The tubes were then incubated at 37 C. and examined at 24, 48, 72, and 96 hours after inoculation for the presence or absence of CP effects (table 4).

Since both the California and Colorado isolates caused clinical IBR and since convalescent serums from calves in which the disease had been produced by inoculation of nasal washings neutralized each of the isolates, final proof was provided that the virus causing IBR had been isolated and identified and that the isolates were identical.[11]

DISCUSSION

When the California workers began studying this disease, it was thought that it might be one of several entities including malignant catarrhal fever, virus diarrhea, mucosal diseases, or even an exotic disease, such as ephemeral fever or bovine influenza. However, in all of these diseases, the infectious agent is associated with the blood or tissues or both. When it was shown that the etiological agent of IBR was present in the nasal secretions only, this feature served largely to differentiate it from the diseases listed above. Finally, the isolation from infected cattle of a hitherto undescribed virus that proved to be the cause of the infection established IBR as a new disease entity.

Reports that IBR has been transmitted by the parenteral and intratracheal inoculation of febrile serums and spleen from dead animals were not confirmed by using materials from cases of IBR in Colorado as well as in California. Attempts to recover virus in tissue culture from visceral organs of infected cattle were likewise unsuccessful. There was no difficulty, however, in reproducing the disease in approximately 45 calves and young cattle by means of nasal secretions from both naturally occurring and experimentally produced cases of IBR or in isolating a number of strains of the virus in tissue culture from such materials. These discrepancies might be attributed to the fact that the disease is contagious and that, unless strict precautionary measures are taken, infection by exposure can readily occur, particularly if the animals are maintained in close contact.

Since certain features of the naturally occurring infection have been observed to occur with great consistency, some conclusions can be drawn regarding the epizootiology of the disease. The fact that initial outbreaks usually occur after the introduction of new cattle strongly indicates that the infection is introduced by subclinically infected or carrier animals; experimentally, it has been found that the infection is sometimes so mild it could be readily overlooked under field conditions. While there is no direct proof that clinically recovered animals may harbor the virus, young susceptible cattle usually become infected shortly after being brought to a place where the disease has recently occurred, which suggests that such a possibility exists.

TABLE 4—Reciprocal Cross-Neutralization Studies in Tissue Culture of the California and Colorado Isolates

Virus	Serum dilutions	Serums: IBR (Calif.) Preinfection	IBR (Calif.) Convalescent	IBR (Colo.) Preinfection	IBR (Colo.) Convalescent
California isolate (10^2 t.c.i.d.$_{50}$)	1:5	8/8*	0/8	8/8	0/8
	1:10	8/8	0/8	8/8	0/8
	1:100	8/8	8/8	8/8	8/8
	1:1,000	8/8	8/8	8/8	8/8
Colorado isolate (10^2 t.c.i.d.$_{50}$)	1:5	8/8	0/8	8/8	0/8
	1:10	8/8	0/8	8/8	0/8
	1:100	8/8	8/8	8/8	8/8
	1:1,000	8/8	8/8	8/8	8/8

* Number of tubes showing cytopathogenic changes/number of tubes inoculated.

The fact that the disease can not always be traced to the recent introduction of cattle, and that it sometimes appears simultaneously in herds on unadjoining properties in the same district, suggests that the virus is normally present in the respiratory tract of some cattle, and that under appropriate stimulus its disease-producing potential is activated. A possible relationship between susceptibility and feeding of the fattening ration used for feeder cattle was suggested by Chow *et al.*[20] However, since the virus could not be found in clinically normal animals, it seems quite unlikely that it is widely distributed in the cattle population.[9]

The disease has occurred thus far only in large concentrations of cattle, such as those found in feedlots and in areas where intensive dairy operations are conducted. This might suggest that the virus must

pass in rapid succession through many animals before it becomes sufficiently virulent to produce a clinical infection. Some basis for this possibility is found in the fact that the experimentally produced infection becomes more severe, up to a point, on serial passage. If this situation exists under field conditions, it would explain why the disease is not observed in small, isolated herds; presumably, there would be too few animals for the virus passage to attain the virulence necessary to produce clinical infection. This would also account for the relatively low morbidity reported for some large herds in which only part of the cattle showed clinical infection; all or most of the remainder may have suffered subclinical infection. However, only large scale serological testing will answer such speculation.

The fact that a solid immunity, lasting at least six months, is acquired on recovery from the experimental infection suggests that artificial means of active immunization might be feasible. Preliminary studies along these lines appear highly promising. The possibility of preparing an effective antiserum from convalescent or hyperimmune cattle serum for prophylactic purposes also appears to offer an additional means of control.

In the absence of any specific immunizing product, the only way any degree of control might be achieved at present would appear to be in segregating the clinically ill animals and treating them with appropriate antibiotics to control secondary bacterial invaders. More specific management recommendations await further clarification of the epizootiology of the disease.

SUMMARY

The clinical and epizootiological features of infectious bovine rhinotracheitis (IBR), a newly recognized virus disease of cattle, are described.

The experimental work by which IBR was established as a disease entity is reviewed.

1) Immunological studies showed that three respiratory conditions (an acute upper respiratory infection of California dairy cattle, a respiratory disease of California feeder cattle, and rhinotracheitis of Colorado feeder cattle) are one disease entity, which was named IBR.

2) Virus isolates recovered from each of three syndromes were proved, by immunological studies in calves, to be identical.

3) Pathogenicity and serological studies proved the isolated virus to be the causative agent of IBR.

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The Infectivity of Blood from Anaplasma-Infected Cattle as Shown by Calf Inoculation

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MANY INVESTIGATORS have conducted studies on the transmission of bovine anaplasmosis; however, most were primarily concerned with the means of transmitting the disease rather than the amount of blood necessary to produce anaplasmosis in a susceptible animal. Many reports relate to the accidental transmission of anaplasmosis by unclean instruments, needles, et cetera, contaminated with infected blood.[1-8] Clinical cases of anaplasmosis have proved to be the most fruitful source of infective material in experimental attempts to demonstrate transmission by insect vectors, such as flies and mosquitoes.[9,10] While such studies have indicated success in transmitting anaplasmosis with small quantities of infective blood, meager information is available on the relative infectivity of such blood in terms of minimum doses.

This study was conducted to determine (1) the relative infectivity of blood from cattle in the acute and carrier stages of the disease by titrations in susceptible animals and (2) the correlation between this infectivity and the complement-fixing titer of the infected animal's blood serum.

EXPERIMENTAL PROCEDURES

Four Anaplasma-infected cattle were used as the source of infective blood. They were selected on the basis of their serum antibody titers as revealed by the complement-fixation (CF) test.[11] They represented 3 carriers and 1 animal with an acute case of the disease. The status of these animals is indicated and the dilution of blood inoculated into each calf in 1.0-ml. doses is shown (table 1).

Animal 3373 was a 6-year-old Hereford steer which was received in June, 1950, from Florida. It was splenectomized in April, 1951, preparatory to anaplasmosis experimental work. Subsequent to splenectomy, bodies in the red blood cells typical of *Anaplasma marginale* appeared, indicating previous infection. The animal has since been retained in isolation as a source of infective material.

Animal 2552 was a 15-year-old, Shorthorn, splenectomized steer, which was experimentally infected on July 10, 1941, with material containing *A. marginale* that had originally been obtained from a natural case of the disease in Delaware. A number of positive subinoculations into susceptible animals had previously been made, using 10.0 ml. or more of citrated blood from these 2 carrier animals.

Animal 3763 was a 2-year-old, Holstein-Friesian, splenectomized steer, which was infected on Dec. 21, 1954, with *A. marginale* constituting the second passage from animal 3373. This animal had shown a maximum of 32 per cent parasitized erythrocytes during the acute stage of the disease. Twelve months following inoculation, blood was collected for this study. Marginal bodies were not observed, nor were acute symptoms present.

Animal 3836 was a 1-year-old, splenectomized, Holstein-Friesian steer inoculated on April 1, 1955, with infective material originating from 2552. Eighteen days after inoculation, this animal was bled for a source of infective blood for this study. At this time, the animal was in the acute stage of anaplasmosis and 30.8 per cent of its red blood cells contained marginal bodies. The red blood cell volume was 18.0 per cent. Blood from this acutely affected animal was injected into two groups of test calves. Group 1 was composed of calves in which the presence of Hemobartonella had been observed following splenectomy and prior to inoculation. Group 2 consisted of splenectomized calves in which no Hemobartonella had been observed.

Whole blood from each donor was collected aseptically into a flask containing sodium citrate sufficient to make a final concentration of 0.5 per cent. The citrated blood was immediately diluted with sterile, normal bovine serum in tenfold serial dilutions. The time elapsing between collection of the blood and inoculation into the calves was less than 30 minutes. All inoculations were made subcutaneously. The animals used for the infectivity tests were dairy-type calves, uniform in size, weight (about 500 lb.), and age. All were splenectomized, castrated, and free of external parasites prior to inoculation. Temperatures were taken and recorded twice daily, starting immediately after splenectomy and continuing for 90 days thereafter. Blood samples were collected several times before and three times weekly following inoculation for a period of 60 days.

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Presented before the Combined Sections on Poultry and Research, Ninety-Third Annual Meeting, American Veterinary Medical Association, San Antonio, Texas, Oct. 15–18, 1956.

The authors acknowledge the advice and counsel of Dr. L. O. Mott in the preparation of this manuscript.

Blood films were prepared from each sample, stained with Giemsa stain, and examined microscopically for the presence of *A. marginale* and Hemobartonella. Red blood cell volume determinations were made on each blood sample immediately following collection by a high-speed centrifugation method.[12]

Serum samples were also prepared from each bleeding and tested by CF for the presence of anaplasmosis antibodies by the method previously described.[11] All animals were carefully checked for the presence of Hemobartonella starting at the time of splenectomy and continuing throughout the experiment. Observations were made for a period of 60 days following inoculation.

The test calves which did not develop anaplasmosis within the 60-day observation period were challenged with virulent inoculums in order to determine their susceptibility. The challenge inoculums were given subcutaneously and consisted of 0.5 ml. of packed blood cells per pound of body weight collected from an animal with acute anaplasmosis having approximately 50 per cent of the red cells parasitized.

RESULTS AND DISCUSSION

The development of bodies characteristic of *A. marginale* in the red blood cells, together with a positive CF reaction and typical clinical symptoms, were used to indicate the presence of infection in the test calves. The incubation periods in those animals which developed anaplasmosis (table 2) represent the number of days following inoculation when marginal bodies and positive CF reactions first appeared. Lotze [13] observed that there is a correlation between the number of parasitized red blood cells injected and the length of incubation period.

Donor animals 3373, 2552, and 3763 were carriers of anaplasmosis, and their red blood cells did not contain marginal bodies in sufficient quantities to make a diagnosis. Their serums did contain CF antibodies with titers of 1:10, 1:40, 1:160, respectively.

The highest dilution of blood from animal 3373 producing anaplasmosis in a test calf was 10^{-1}. Marginal bodies first appeared on the thirty-seventh day; however, the positive CF reaction began on the thirtieth day following inoculation. The test calf which received 1.0 ml. of undiluted blood developed marginal bodies nine days sooner than the test calf which received 10^{-1}. Also positive CF reactions appeared seven days sooner in the calf receiving undiluted blood.

The highest dilution of blood from carrier animal 2552 producing anaplasmosis in a test calf was 10^{-2}. Marginal bodies first appeared on the twenty-ninth day, and positive CF reaction began on the twenty-seventh day after inoculation. In the 2 animals in this series, which received larger quantities of infective blood, marginal bodies appeared after 24 and 20 days, respectively, whereas positive CF reactions appeared in 20 and 17 days, respectively.

The highest dilution of blood from carrier animal 3763 producing anaplasmosis in a test calf was 10^{-3}. Marginal bodies

TABLE 1—The Donor Animals and Dilutions of Their Blood Used for Calf Inoculations

Infected donor animal and status	Period infected	Culture identification and passage No.	Complement-fixation serum titer of donor animal	Dilutions of blood* (1-ml. dose)
3373, carrier	4½ yr. known	Florida 2 Original field case	1:10	Undiluted 10^{-1} 10^{-2} 10^{-3} 10^{-4}
2552, carrier	14 yr.	Delaware 1 Passage 2	1:40	Undiluted 10^{-1} 10^{-2} 10^{-3} 10^{-4}
3763, carrier	1 yr.	Florida 2 Passage 2	1:160	10^{-1} 10^{-2} 10^{-3} 10^{-4}
3836, acute	18 days	Delaware 1 Passage 3	1:320	10^{-2}† 10^{-4}† 10^{-5}† 10^{-6}† 10^{-7}†

* One milliliter of each dilution was inoculated into a splenectomized calf. † This dilution was inoculated also into a splenectomized calf infected with Bartonella.

first appeared on the fifty-first day, and positive CF reactions began on the forty-second day after inoculation. One of the other 2 infected animals in this series had approximately the same incubation period, while the incubation period of the second was somewhat shorter. This series of animals was observed to have a considerable number of pleomorphic rods in their blood typical of Hemobartonella.

The infectivity of blood from the animal with acute anaplasmosis (3836), having a CF serum titer of 1:320, was determined in two series of calves. The highest dilution of blood which produced anaplasmosis in group 1 was 10^{-6}. The other 3 test calves which received a larger quantity of infective blood also developed anaplasmosis. The incubation periods for these 4 animals were from 29 to 43 days following inoculation. The days following inoculation when positive CF reactions were first observed were either on, or shortly before, the days when marginal bodies first appeared in the blood.

The highest dilution of blood from the animal with an acute infection (3836) which produced anaplasmosis in group 2 was 10^{-5}. The incubation periods of 3 test calves in this group was from 24 to 31 days. The relative infectivity titers of the 3 carriers and 1 animal with an acute case of anaplasmosis are shown (fig. 1).

From these comparative data, it is apparent that a close relationship exists between the amount of the agent in the blood and the serum antibody titers revealed by the CF test. The maximum percentage of parasitized red blood cells and the minimum red blood cell volumes observed during these studies are shown (table 3). The test calves which became infected from

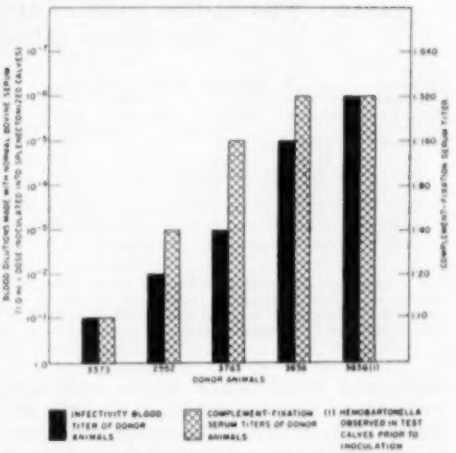
DONOR ANIMALS
INFECTIVITY BLOOD TITER OF DONOR ANIMALS
COMPLEMENT-FIXATION SERUM TITERS OF DONOR ANIMALS
(1) HEMOBARTONELLA OBSERVED IN TEST CALVES PRIOR TO INOCULATION

Fig. 1—Correlation between the infectivity blood titer and the complement-fixation serum titer of cattle infected with anaplasmosis.

carrier blood exhibited clinical reactions of varying degrees. Those calves receiving the larger quantity of infective blood developed the highest percentage of parasitized cells. This same general trend occurred in the test calves inoculated with blood from the animal with acute anaplasmosis. All calves infected with anaplasmosis developed moderate to severe anemia as revealed by their red blood cell volumes.

Of the 9 test calves which did not develop indications of anaplasmosis, 7 were challenged with virulent material containing *A. marginale*. The results of the challenge are shown (table 3). These challenge inoculums were proved in susceptible animals to produce acute anaplasmosis, resulting in 40 to 80 per cent of the cells having marginal bodies. Six of the 7 calves

TABLE 2—Incubation Periods of Anaplasmosis in Splenectomized Calves

Dilutions of blood (1-ml. dose)	Carrier case 3373 (1:10)*		Carrier case 2552 (1:40)*		Carrier case 3763 (1:160)*		Acute case 3836 (1:320)*		Acute case 3836 (1:320)*	
	A. marginale†	CF‡	*A. marginale*†	CF‡	*A. marginale*†	CF‡	*A. marginale*†	CF‡	*A. marginale*†	CF‡
Undiluted	28	23	20	17	....	....	Group 2		Group 1	
10^{-1}	37	30	24	20	49	42	....	....	....	....
10^{-2}	—§	—	29	27	40	44	....	....	....	....
10^{-3}	—	—	—	—	51	42	24	24	29	24
10^{-4}	—	—	—	—	—	—	27	27	34	31
10^{-5}	....	....	....	....	—	—	31	29	43	43
10^{-6}	....	....	....	....	....	....	—	—	36	34
10^{-7}	....	....	....	....	....	....	—	—	—	—

* Anaplasmosis complement-fixation (CF) serum titer. † Number of days after inoculation when marginal bodies first appeared. ‡ Number of days after inoculation when positive CF reaction first appeared. § — = negative reaction.

TABLE 3—Blood Studies on Splenectomized Calves Inoculated with Anaplasma-Infected Blood

Dilutions of blood (1-ml. dose)	Carrier case 3373 (1:10)*		Carrier case 2552 (1:40)*		Carrier case 3763 (1:160)*		Acute case 3836 (1:320)*		Acute case 3836 (1:320)*	
	A. marginale†	RCV‡	*A. marginale*†	RCV‡	*A. marginale*†	RCV‡	*A. marginale*†	RCV‡	*A. marginale*†	RCV‡
Undiluted	27.0	14.0	24.8	8.0	.....	.....	Group 2		Group 1	
10^{-1}	15.4	12.5	11.0	6.0	21.0	20.0	.....	.....	.....	.....
10^{-2}	—	17.0§	5.9	16.0	18.0	22.0	.....	.....	.....	.....
10^{-3}	—	26.0§	—	22.0	13.6	15.0	30.0	11.5	18.0	10.0
10^{-4}	—	24.0	.....	.....	—	19.0	29.3	9.0	24.0	13.5
10^{-5}	.....	.....	.....	.....	—	32.5§	22.8	8.0	10.0	14.3
10^{-6}	.....	.....	.....	.....	.....	.....	—	27.0§	17.5	10.0
10^{-7}	.....	.....	.....	.....	.....	.....	—	24.0§	—	22.0§

* Anaplasmosis complement-fixation serum titer. † Maximum percentage of red blood cells affected with *Anaplasma marginale*. ‡ Minimum percentage of red blood cell volume. § Animals resistant to challenge with blood from an animal in the acute stage of anaplasmosis. ‖ Animals susceptible when challenged with blood from an animal in the acute stage of anaplasmosis.

challenged developed a severe infection, with 42 to 69 per cent of their cells affected. One developed a rather mild case in which only 5.8 per cent of the red blood cells were affected. This result is believed to indicate a degree of resistance in this calf, even though it did not show clinical infection from the original inoculum of diluted blood.

SUMMARY

The infectivity titration of blood collected from 3 cattle in the carrier state of anaplasmosis revealed that the animals with complement-fixing serum titers of 1:10, 1:40, and 1:160 had blood-infectivity titers of 10^{-1}, 10^{-2}, and 10^{-3}, respectively. The blood from the animal with acute anaplasmosis had a serum titer of 1:320 with the complement-fixation (CF) test, whereas blood from this animal had an infectivity titer of 10^{-5} in one group and 10^{-6} in the other group of splenectomized calves. These results on blood collected from anaplasmosis-infected cattle indicate that the animals whose serums showed a high CF titer also had a relatively high infectivity titer, whereas those animals with a low CF titer had a much lower degree of infectivity.

In general, the length of the incubation periods was progressively longer and the maximum percentage of parasitized cells was progressively smaller for each tenfold dilution of the inoculum.

The positive CF reactions in 14 of the 15 test calves which developed anaplasmosis appeared either on or up to nine days (average 3 days) before the appearance of marginal bodies.

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Normal Development of the Germinal Epithelium and Seminiferous Tubules in the Bull

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ALTHOUGH considerable progress has been made in the field of physiology of reproduction in domesticated animals, little is known about the normal development and histology of the bovine testis. With the introduction of artificial insemination, bull fertility has become more and more important, and extensive studies of the histological elements of the testis have been made in young and adult animals. However, a thorough study of the histological picture of prenatal and immature bovine testis has not yet been made. This paper reports the results of a study of the normal development of the germinal epithelium and seminiferous tubules in prenatal and immature bulls.

LITERATURE REVIEW

It is not within the scope of this review to discuss the two controversial points of view concerning the origin of germ cells, since this investigation begins with the study of already organized sex cords. Investigators agree on the point that the sex cords, from very early embryonic life, contain two types of cells: the large cells and the small cells. Both types are clearly distinguished throughout the embryonic and fetal development of the testis. The point of controversy, however, is over the role that these cells play in spermatogenesis.

It is important to review the papers which deal with these cells, since they are emphasized throughout this study. In the testis of very young and growing mammals, Biondi [4] found only one kind of cell. Germano [11] reported that the seminiferous tubules of both the dog and cat at birth had only one type of cell, a few of which had begun to undergo transformation toward spermatogonia. Rubaschkin,[28] however, described large and small cells in the testes taken from embryos of the guinea pig, dog, and cat, and Hargitt [15] proved the existence of both kinds of cells in the testis of the cat a short time after birth. Allen [1] did not find any large cells in the testis of the albino rat seven days after birth.

Hargitt [16] claims that all embryonic testes of mammals thus far examined contain both large and small cells. According to him, in the rat the large cells divide by mitosis and decrease in number until about the ninth day after birth, by which time all large cells disappear from the solid seminiferous tubules.

Another point of discussion is whether or not the large cells are primordial cells that give origin to the definitive germ cells. Nonidez,[24] who worked with the testes of hen-feathered cocks, pointed out that spermatogonia and many of the columnar epithelial cells disappear without leaving any trace of their former existence. Semon [30] and Swift,[31] working with birds, indicated that the large cells give rise to the definitive germ cells. On the other hand, Firket [6] and Gatenby,[10] working with similar material, obtained negative results.

The same situation has been found to exist in mammals. A number of investigators have reported that the large cells give rise to definite spermatogonia. Arey [2] pointed out that the large cells are stem cells which proliferate and become spermatogonia while the smaller cells are indifferent cells. The most complete works on the subject, however, indicate that the large cells have nothing to do with the definitive germinal epithelia, and Hargitt [19] expressed the conviction that this phenomenon is generally characteristic of mammals. Recently, however, Kirkpatrick,[19] working with fox squirrels, mentioned a possible transformation of these large cells into primary spermatocytes.

There are few studies concerning the normal development of the bovine testis. A majority of the papers, such as those of Lillie,[21] Bascom,[3] Bissonnette,[5] and Hooker,[18] are based on studies of the interstitial tissue. According to Wodsedalek,[35] spermatogenesis in the bull generally corresponds to that of the boar and stallion.

Masui [22] claimed that amitotic division is frequently found in the earliest generation of spermatogonia in cattle. Wodsedalek,[35] however, did not report such a division in spite of having studied the testes of small embryos, 1 fetus about 6 months old, and mature bulls. The excellent paper of Schoenfeld [29] should be referred to by those interested in the cytology of the bovine testis. Phillips and Andrews [26] described briefly the histological picture of the bovine testis. Fossland [9] noted a fourfold increase in the diameter of the seminiferous tubules between the first and eighteenth month. Spermatozoa were not observed in the lumens of the seminiferous tubules until the ninth month in Jerseys and the tenth month in Holstein-Friesians.

Paper of the journal series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Dairy Industry, New Brunswick.

TABLE 1—Animal Weight, Testes Weight, and Diameters* of Seminiferous Tubules

Breed	Age (days)	Body weight (lb.)	Testes (both) weight (Gm.)	Diameters of seminiferous tubules		
				Average (μ)	Maximum (μ)	Minimum (μ)
Holstein-Friesian	90 fetus	.....	0.5	34.3	59.0	27.0
Holstein-Friesian	120 fetus	1.4	0.76	49.8	57.0	36.0
Holstein-Friesian	180 fetus	.....	1.5	43.5	51.0	39.1
Guernsey	190 fetus	.....	1.4	42.8	50.7	38.6
Brown Swiss	200 fetus	18.4	1.4	30.6	34.8	24.7
Holstein-Friesian	1 calf	79.0	8.0	42.3	51.0	34.1
Holstein-Friesian	1 calf	81.0	8.7	41.6	50.6	33.4
Guernsey	14 calf	74.0	6.6	39.8	44.2	34.0
Jersey	16 calf	72.0	8.0	46.1	59.5	37.4
Guernsey	18 calf	76.0	7.8	54.1	61.2	42.1
Holstein-Friesian	20 calf	122.0	8.0	49.5	59.5	37.4
Guernsey	20 calf	81.0	8.8	57.1	68.0	49.3
Jersey	35 calf	83.0	10.3	59.5	68.0	47.6
Jersey	42 calf	78.0	14.0	47.4	56.1	42.5
Guernsey	43 calf	96.0	14.0	49.0	59.5	42.3
Holstein-Friesian	45 calf	.....	19.0	50.1	59.5	44.2
Jersey	45 calf	77.0	17.0	50.3	59.5	42.5
Jersey	70 calf	142.0	14.0	64.9	76.5	57.0
Guernsey	70 calf	150.0	15.0	65.8	78.0	57.9
Jersey	71 calf	131.0	17.4	51.4	62.3	43.3

* The figures were obtained by averaging the diameters of 20 tubules measured at x 450 magnification.

MATERIALS AND METHODS

The material for this investigation consisted of 21 animals, 5 prenatal and 16 postnatal. The age of some of the postnatal calves not belonging to the experiment station was determined according to Sisson and Grossman[37] and Sanz-Egana.[29] The age of the fetuses was determined according to Hammond[13] and Winters *et al.*[34] In 1 animal, testicular biopsies were performed under local anesthesia.

The testes were removed soon after the animals were killed and immediately dissected and weighed on a torsion balance. Small pieces were cut to allow prompt fixation. After the tissues had been in the fixative for four or five hours, they hardened slightly and it was possible to cut the pieces into smaller blocks—none more than 0.5 cm. This permitted a better fixation of the tubules without disturbing the normal histological elements.

Fixatives used were: Bouin's fluid, Flemming's and Zenker's, and 10 per cent formalin. The stains employed were: Cajal's triple stain, Delafield's hematoxylin, iron-hematoxylin, Harris' hematoxylin, eosin, orange G, gentian violet, and fuchsin followed by picric acid. Delafield's hematoxylin and eosin were found to be the most

TABLE 2—Total Number of Cells Contained in 20 Transversal Sections of Seminiferous Tubules*†

Animal	Large cells				Small cells			
	Resting stage	Dividing stage	Degenerating stage	Total	Resting stage	Dividing stage	Degenerating stage	Total
HF– 90 fetus	....	....	....	48	....	....	....	203
HF–120 fetus	45	20	0	65	145	134	3	282
HF–180 fetus	28	5	0	33	143	109	0	252
G–190 fetus	23	9	0	32	138	102	0	240
BS–200 fetus	18	11	0	29	190	60	0	279
HF– 1 calf	10	7	15	32	232	50	26	308
HF– 1 calf	9	8	13	30	229	52	22	303
G– 14 calf	15	12	14	41	179	150	16	345
J– 16 calf	11	4	16	31	127	149	17	293
G– 18 calf	19	9	18	46	248	243	9	500
HF– 20 calf	10	11	18	39	110	278	9	397
G– 20 calf	17	22	9	48	205	207	4	416
J– 35 calf	28	20	19	67	229	283	3	515
J– 42 calf	7	10	10	27	193	215	4	412
G– 43 calf	23	11	13	47	379	231	0	610
HF– 45 calf	5	2	18	25	445	75	20	540
J– 45 calf	5	5	14	24	328	144	42	514
J– 70 calf	35	5	30	70	503	99	6	608
G– 70 calf	29	6	31	66	487	103	10	600
J– 71 calf	21	4	27	52	473	108	5	586

* When primary spermatocytes or spermatogonia in growth period were found, they were counted as large cells.

†G, HF, J, and BS stand for Guernsey, Holstein-Friesian, Jersey, and Brown Swiss, respectively. The number stands for age in days.

satisfactory stains for general purposes. Paraffin sections were cut at 5 to 10 μ; those cut at 6 μ were found to be sufficiently thin. When there was not sufficient time to employ a lengthy method, excellent results were obtained by using the benzene and acetone method of Krajian [20] and Hammack [12] or Cajal's triple stain method.[5]

Measurements were taken with an eyepiece micrometer scale which was calibrated with a stage micrometer at x 450. The measurements of the diameter of the seminiferous tubules represent in every case the average measurements of 20 seminiferous tubules, ten taken from the region near the tunica albuginea and ten from the rete-testis region. The diameter of the seminiferous tubules was measured from basement membrane to basement membrane. Only tubules of a perfectly clear transversal section were measured, and germinal cells found in transversal sections of the seminiferous tubules were counted.

All photomicrographs were taken from sections stained with Delafield's hematoxylin and eosin.

Results

1) *Prenatal Animals.*—A fetus of about 90 days had testes weighing 0.5 Gm. and seminiferous tubules with an average diameter of 34.3 μ (table 1). In a cross section of one seminiferous tubule, the average number of cells was 13. At this stage, the sex cords had become definitely established, being separated from each other by mesenchymal tissue. However, these syncytial masses did not occupy all the spaces between the sex cords. The intertubular cells of the stroma were very prominent. Within the sex cords, there were two kinds of cells, the large and the small cells, which were irregularly scattered.

The testes of a 120-day-old fetus weighed 0.76 Gm. The average diameter of the seminiferous tubules was 49.8 μ (table 1). The average number of cells contained in a cross section of one tubule was 17 (table 2). At this stage, the testis contained well-developed tubules (fig. 1 to 3). There was abundant intertubular tissue and more distinct separation of the sex cords (fig. 2); however, the cords remained infantile without lumens. Within the cords, the small cells formed a layer along the basement membrane. These cells were irregular in shape and their nuclei were ovoid, round, or elongated (fig. 3). Some of these cells were in a state of amitotic division. The large cells appeared to arise from the basement layer and rapidly increased to about twice their size. It was frequently observed at this stage that a single cell occupied almost the entire diameter of the cord.

The weight of the testes of a 180-day-old fetus was 1.5 Gm. The sex tubules had an average diameter of 43.5 μ (table 1). The average number of cells contained in a cross section of one tubule was 14 (table 2). About the same testicular development was found in a 190-day-old fetus (table 1). The seminiferous tubules were well established, narrow, and more convoluted, and an abundant stroma isolated one tubule from another. This mesenchymal tissue was packed densely between the radiating tubules. The lumen of the rete testis had reached the inner end of the tubules. The large cells occurred in almost all tubules, and division was frequent. The nuclear organization of these cells and their cytoplasmic structure resembled the defini-

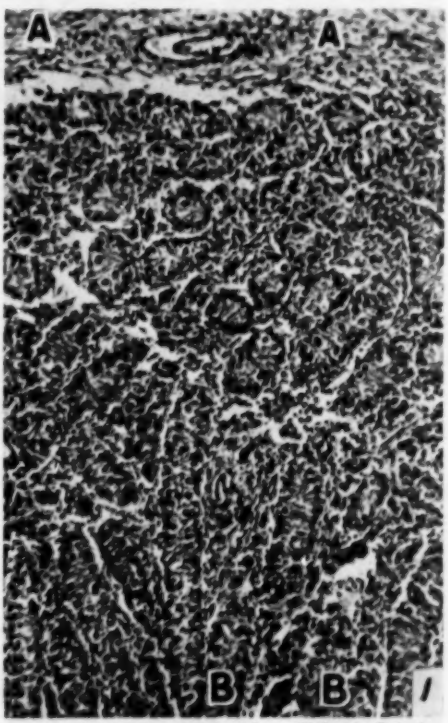


Fig. 1—Photomicrograph of a Holstein-Friesian fetal testis about 120 days old. The well-defined seminiferous tubes are immersed in a mass of mesenchymal tissue. (A) Tunica albuginea. (B) Part of the rete testis.
Hematoxylin-eosin stain. Section, 5 μ. x 120.

tive spermatogonia. The small cells had the same characteristics as those of previous stages and no degenerate cells appeared at this stage.

The weight of the testes of a 200-day-old fetus was 1.4 Gm., and the seminiferous tubules had an average diameter of 30.6 μ (table 1). The average number of cells

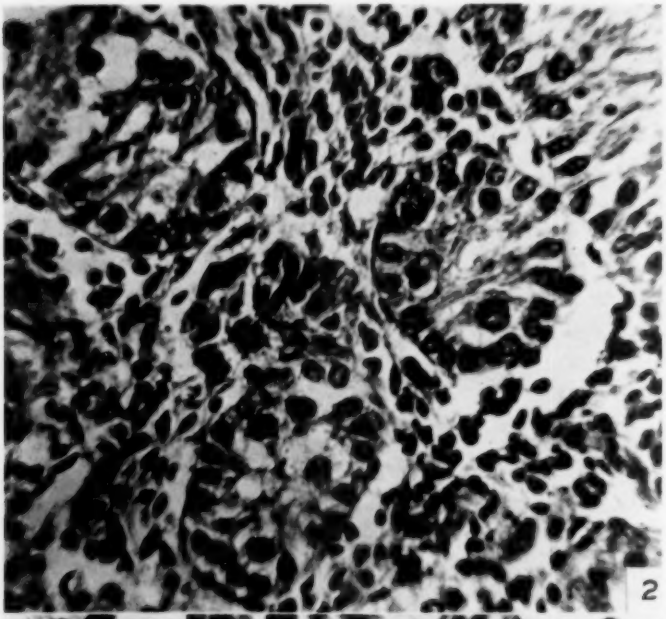


Fig. 2—Same section as in figure 1, showing the arrangement of the mesenchymal elements and the small and large nuclei of three seminiferous tubules. x 516.

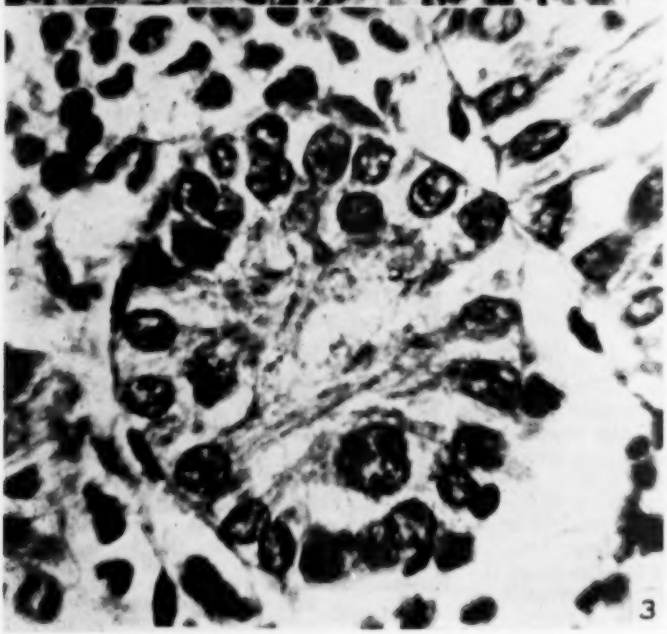
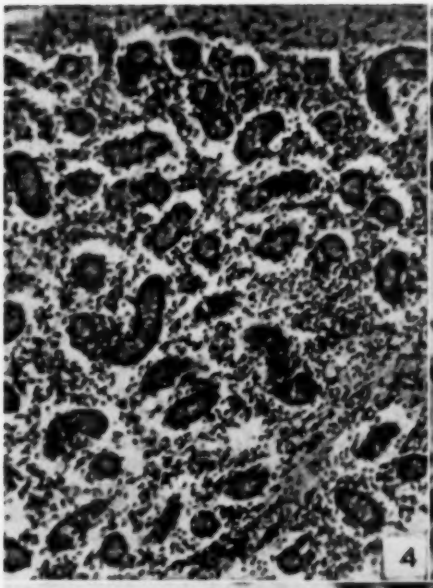
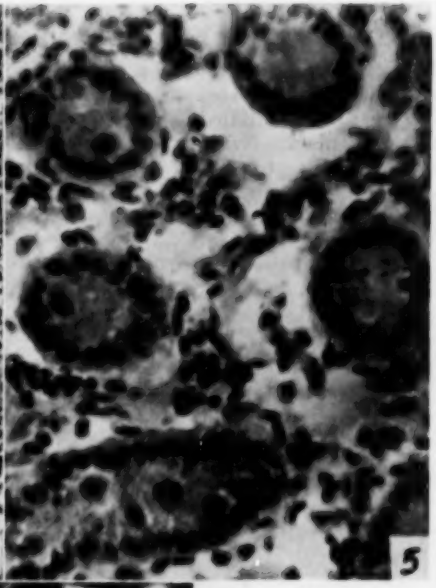


Fig. 3—A detail of the nuclei contained in one of the seminiferous tubes shown in figure 2. x 990.





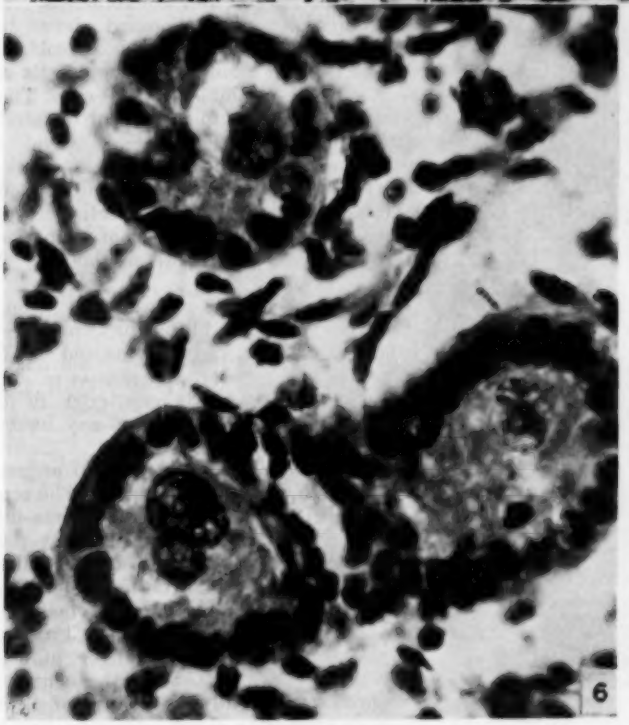


Fig. 4—Photomicrograph of a Brown Swiss fetal testis about 200 days old, showing the general pattern of the seminiferous tubules and interstitial tissue at this stage. The section shows the testis from the tunica albuginea (top) to the rete testis (bottom). Hematoxylin-eosin stain. Section, 6 μ. x 120.

Fig. 5—Photomicrograph of the same section as in figure 4. Large cells appear in all tubules. The mesenchymal cells do not fill the intratubular space uniformly. x 473.

Fig. 6—Photomicrograph of the same section as in figure 4. This figure shows mitosis of large cells. Although the three tubes exhibit nuclear chromatin of large cells, only one tube (bottom, left) shows the stage of division clearly. x 990.

per cross section of one tubule was 15 (table 2). The diameter of the seminiferous tubules of this fetus was the smallest of all the testes studied; otherwise, the histological picture (fig. 4 to 6) of this testis was similar to that of the 2 fetuses described above.

2) *Postnatal Animals.*—At birth, the average weight of the testes was about 8.0 Gm. The seminiferous tubules had an average diameter of 42.0 μ. In a cross section of one seminiferous tubule, the average number of cells was 17. The seminiferous tubules were solid, but they resembled the definitive seminiferous tubules (fig. 7). At this stage, there was a large number of convoluted tubules surrounded by a fairly dense mass of fibroblastic tissue. It was possible to identify typical interstitial tissue although many of the nuclei seemed less chromophilic. The large cells lay toward the center of the cord (fig. 8 and 9). Some of them were undergoing a marked hypertrophy. Frequent division was observed among the large cells. At this stage, giant cells with a great number of chromosomes were noticed, although the chromosomes were smaller than those in cells of normal size. The small basal cells of the tubules were increasing in number by mitosis, and some of them appeared to be dividing by amitosis. The number of small cells that were in a degenerating stage may have been the result of such division.

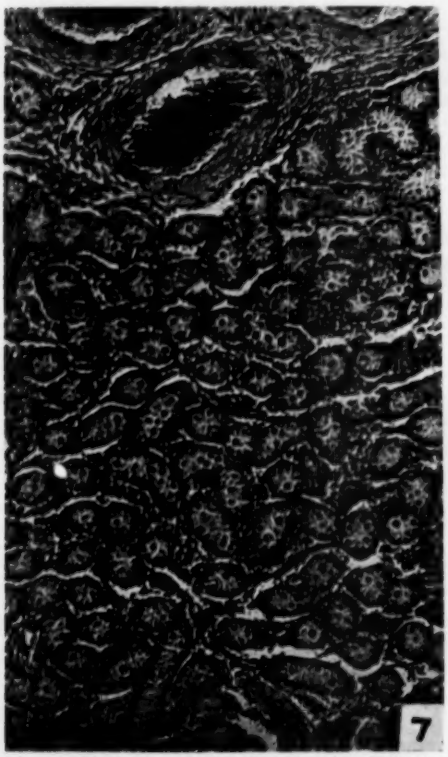


Fig. 7—Photomicrograph of testis of a Holstein-Friesian calf 1 day old, showing the remarkable increase in tubular convolutions when compared with previously studied testes. Large blood vessels are present in the tunica albuginea (top). Hematoxylin-eosin stain. Section, 7 μ. x 120.

Animals about 2 weeks of age showed an average testicular weight of 7.3 Gm., and the average diameter of the seminiferous tubules ranged from 39.8 to 46.6 μ. At this age, the seminiferous tubules decreased slightly in diameter but they increased in length and convolutions. The germinal epithelium remained almost like that of the testis of a newborn calf, the only difference being that some cells appeared which resembled Sertoli cells. These cells were observed to be present in tubules with an active "spermatogenic wave" alternating in amitotic and mitotic division. Many cells, both large and small, had degenerated from the time mitotic division appeared. A large number of giant cells were observed in the testis of this age (fig. 10 and 11).

The 18-day testis showed the ground substance (matrix), filling the seminiferous tubules, to be already segmented in the center of the tubules, and masses of chromatin were frequently scattered in the center of the tubules without any nuclear membrane.

Twenty-day-old testes exhibited an average testicular weight of 8.4 Gm. The seminiferous tubules of these calves presented the same arrangement as those previously described, but they had increased in diameter. The intertubular tissue still showed clearly distinguishable mesenchymal cells, but their protoplasm had become less chromophilic than that of younger animals. The large cells lay toward the center of the tubules in an advanced stage of degeneration (fig. 12); giant cells were present and amitotic divisions were observed (fig. 13). At times, a large cell was observed to have divided by amitosis and, before a

complete separation of the protoplasm took place, both nuclei began a new amitotic division. Other large cells showed a ring of small granules of chromatin bordering the nuclear membrane, while the center of the nucleus was completely clear. The small cells of the basement membrane increased in number by mitosis and two generations of cells could be distinguished, one of them made up of smaller cells than the other.

At 35 days of age, the testes weight was

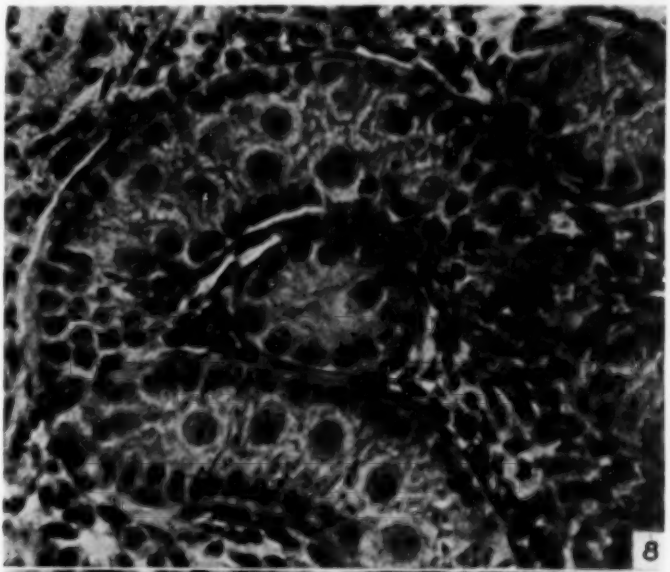


Fig. 8—This photomicrograph of the same section as in figure 7 shows a longitudinal section of a seminiferous tubule. The tubule has no lumen. Large cells fill the center of the tubule. A single layer of small cells lines the basement membrane. The intratubular space is filled by a dense mass of mesenchymal tissue. x 516.

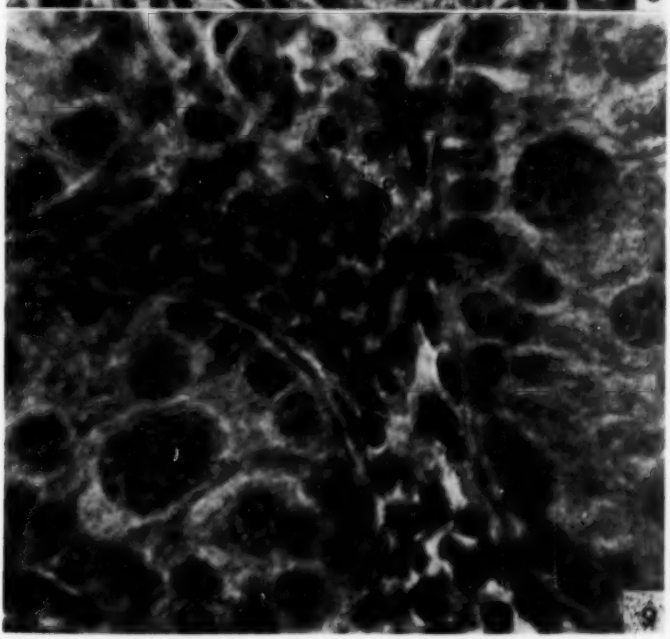


Fig. 9—Same section as in figure 7. A section of three seminiferous tubules, showing the nuclear structure of large cells toward the center of the tubules. x 850.

10.3 Gm. and the average diameter of the seminiferous tubules was 59.5 μ. At this age, the testis had not made any striking advances over the 20-day stage. Some intertubular spaces were filled by mesenchymal cells and some vesicular nuclei which resembled the nuclei of Leydig cells, while others were devoid of cells (fig. 14). The

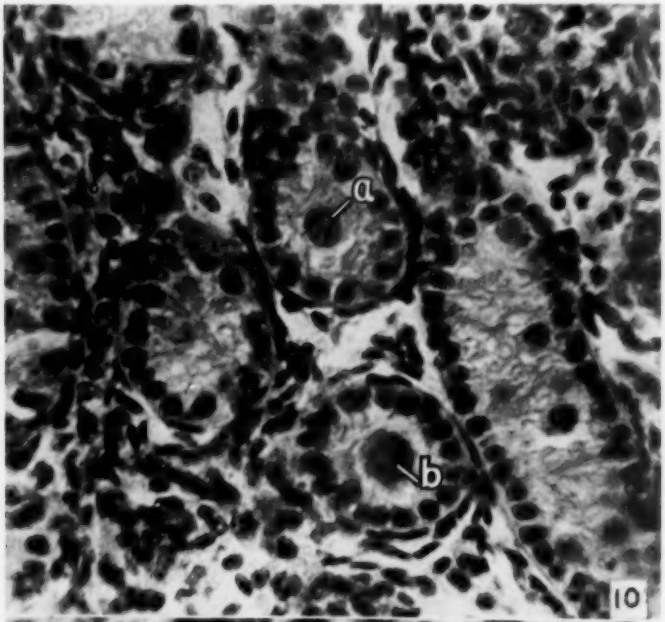


Fig. 10—Photomicrograph of testis of Jersey calf 16 days old. The intratubular matrix toward the center of the tubules contains condensed masses of chromatin (longitudinal section), the remains of large cells, a nucleus of a normal large cell (a), and condensing chromatin of a giant cell (b).

Fusiform and ovoid nuclei are abundant, arranged between the seminiferous tubules.

Hematoxylin-eosin stain. Section, 7 μ. x 516.

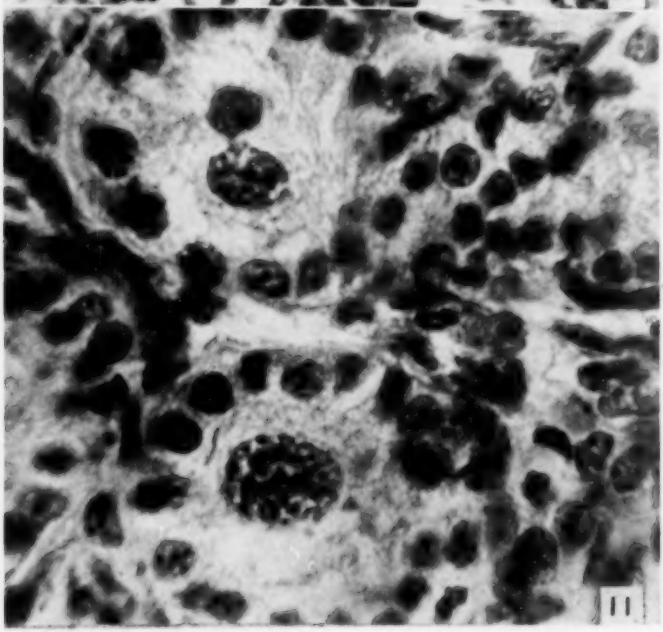


Fig. 11—Another field of the testis shown in figure 10, showing a hypertrophied nucleus in the center of the tubule (bottom). Another large cell is ending amitotic division in the tubule at the top. Hematoxylin-eosin stain. Section, 7 μ. x 990.

tubular wall was more defined, and the ground substance which filled the lumen of the tubule was segmented. The nuclei of the large cells were reduced in size and in number. In many of them, the nuclear chromatin had lost a great deal of its affinity for hematoxylin. All degrees of degeneration were observed in the large cells in this stage. The small basal cells of the tubules showed a marked increase in number and some advanced mitoses were observed.

Calves of 42 to 45 days of age showed a testes weight ranging from 14.0 to 19.0 Gm. Although the animals in this group were of different breeds, they presented

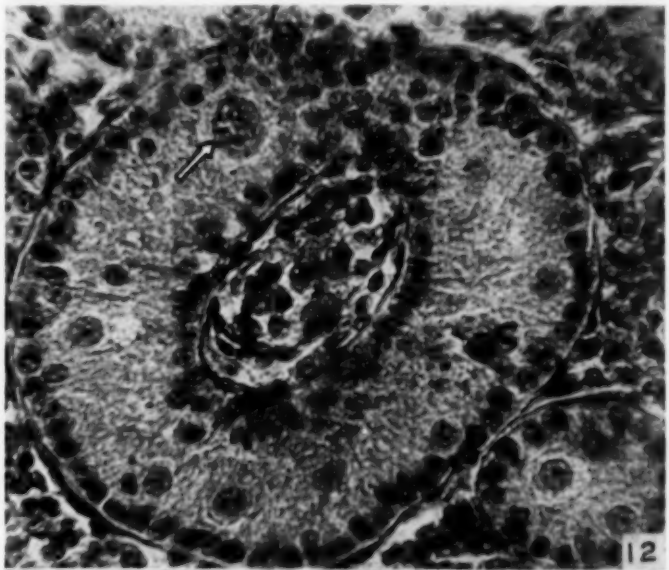


Fig. 12—Photomicrograph of testis of a Guernsey calf 20 days old. Longitudinal section of a seminiferous tubule showing within the tube pyknotic nuclei of large cells, crusts of chromatin, and a giant cell (arrow).

The cells near the basement membrane have increased; while some still have undifferentiated nuclei, others are already differentiated into Sertoli cells and spermatogonia.

Hematoxylin-eosin stain. Section, 8 μ. x 516.

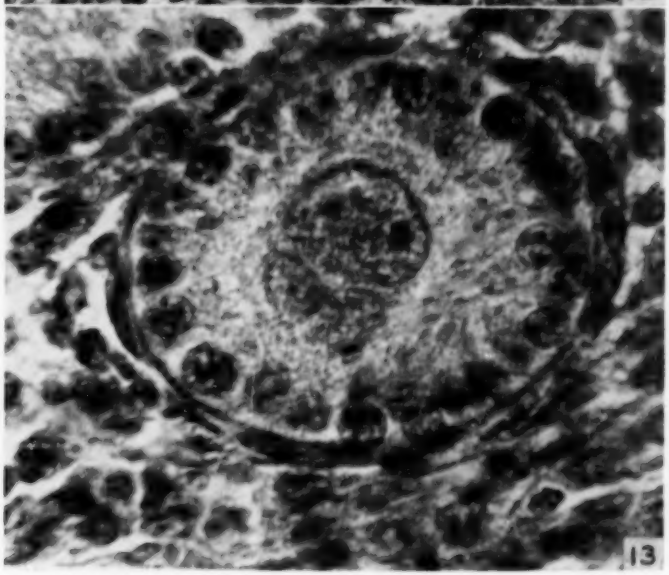


Fig. 13—This photomicrograph of the same section as in figure 12 shows, in the center of the tube, a mass of pulverulent chromatin of a giant cell. Observe that in the process of reabsorption the nuclei splits in two. x 990.

a similar histological picture. The mesenchymal cells were more uniformly arranged; in some sections, the interstitial cells lay in masses arranged in parallel strands. The tubules showed a greater resemblance to functional seminiferous tubules. Large cells disappeared completely from most of the tubules (fig. 15) and almost all of those that remained presented different stages of degeneration. The small cells lay close together on the basement membrane and, in some tubules, started to

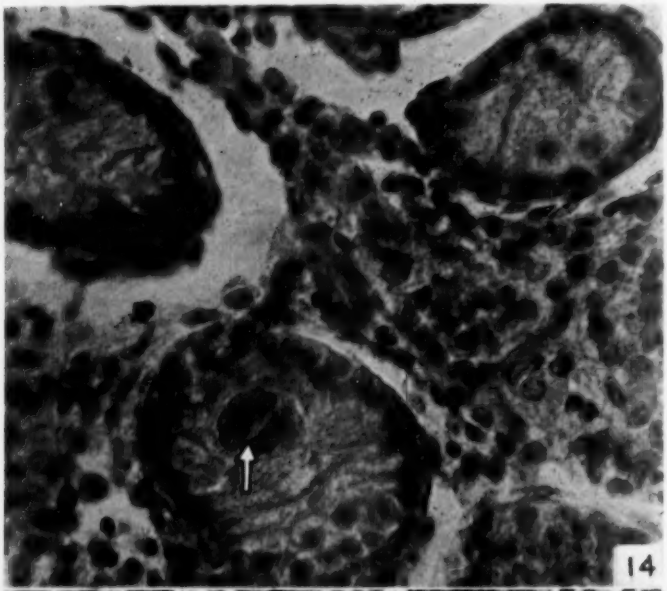


Fig. 14—Photomicrograph of testis of a Jersey calf 35 days old, showing nucleus (arrow) of a large cell degenerating. The chromatin of this nucleus, instead of spreading as pulverulent chromatin (fig. 13), forms two amorphous crusts. The interstitial tissue shows fusiform, ovoid, and round nuclei. Hematoxylin-eosin stain. Section, 7 μ. x 516.

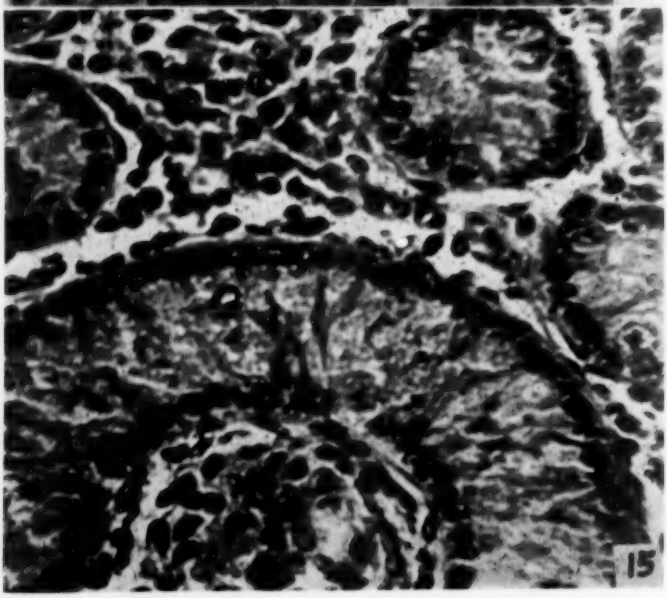


Fig. 15—Photomicrograph of testis of a Jersey calf 45 days of age, showing a longitudinal and three transversal sections of seminiferous tubules devoid of large cells. The interstitial tissue shows the same characteristics as in figure 14. Hematoxylin-eosin stain. Section, 5 μ. x 516.

form two rows of cells which resembled definitive spermatogonia.

The weight of the testes of animals 70 and 71 days of age was about 15.5 Gm., and the average diameter of the seminiferous tubules ranged from 51.4 to 65.8 μ. At this stage, the tubules had increased in diameter, length, and convolutions (fig. 16). Although they appeared to be solid, they seemed to be definitive seminiferous tubules. The intertubular stroma showed that among the mesenchymal cells there were some nuclei which resembled those of Leydig cells, while other cells occurred with intermediate nuclei (fig. 17 and 18). The chief noticeable change was a further increase in the number of cells within the tubule. At this stage, most of the nuclei which lay along the basement membrane were differentiated spermatogonia (fig. 17 and 18), others were still undifferentiated elements, and occasionally Sertoli nuclei were present. The spermatogonia were spherical or ovoid cells (fig. 18) with clear cytoplasm, and their size ranged between 8.0 and 15.0 μ. The nuclear membrane was not always discernible, especially in cells presenting crustlike nuclei.

Rising from the spermatogonia layer, primary spermatocytes appeared for the first time at this stage (fig. 17 and 18). The majority of the nuclei of these cells were clearly distinguishable from their parent cells, the spermatogonia, by their larger size (3/5 to 7/10 larger than the spermatogonia) and because the majority of their nuclei exhibited the characteristic chromosomal filaments.

Some cells were counted as being in the resting stage, but after examination under 1,500 magnifications it was noticeable that the nuclear chromatin was undergoing a reabsorption process or becoming a pulverulent mass within the nuclear membrane. Giant cells and two amitotic divisions were observed. The nuclei near the center of the tubule disappeared either by reabsorption or by nuclear contraction.

The results on an experimental calf are included in this report, since it is believed that they contribute to the subject under consideration. It was relatively easy to trace the development of the germinal epithelium following its destruction by thyroprotein feeding.

From 5 to 22 days of age, this calf was fed 2.0 Gm. of thyroprotein daily. At 64 days of age, the first biopsy was performed and the histological study showed that the average diameter of the seminiferous tubules was 60.3 μ. The average number of cells per cross section of seminiferous tubule was 23 and the intertubular spaces were much larger than the average control. The large cells near the basement membrane were spermatogonia or undifferentiated cells, and more than half of the total number of small cells were in the stage of degeneration. There were also differentiated Sertoli cells, but the most salient histological picture was that of the undifferentiated cells. They exhibited crustlike nuclei that appeared to be joined to the basement membrane (fig. 19).

At 80 days of age, there was no marked difference from the previous biopsy. The average diameter of the seminiferous tubules was 59.3 μ, and the average number of cells per cross section of the seminiferous tubule was 24. The interstitial cells

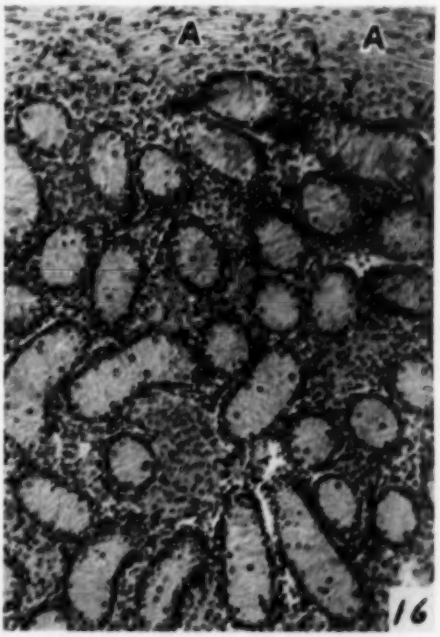


Fig. 16—Section of testis of a Jersey calf 70 days old. The pattern of the histological elements of the testis at this age are shown in this photomicrograph. The large cells within the tubule are mostly primary spermatocytes. (A) tunica albuginea.
Hematoxylin-eosin stain. Section, 5 μ. x 120.

were observed to be in different stages of transformation from mesenchymal cells or fibroblasts to definite Leydig cells. These cells were arranged in the form of intercellular threads among connective tissue fibers. Many of the interstitial cells presented a differentiated cellular membrane with vacuolated nuclei which had two or three nucleoli. The cytoplasm of the center of the tubules was liquefying and some

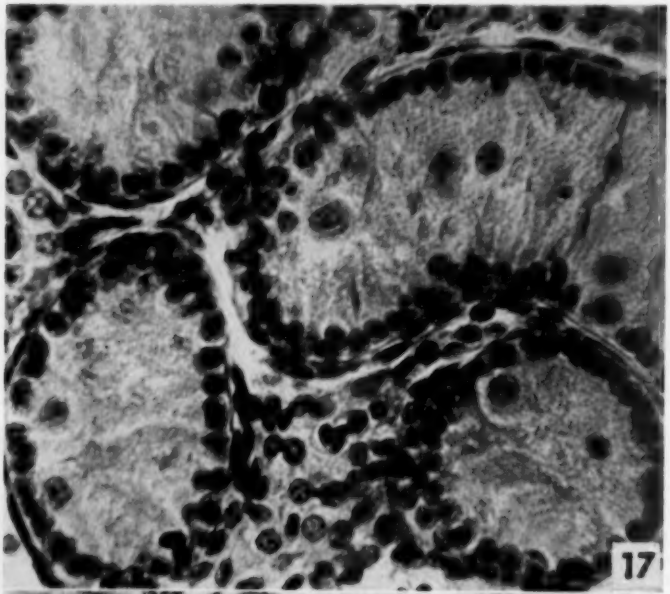


Fig. 17—Same section as in figure 16. This photomicrograph shows the increasing number of cells near the basement membrane and some enlarging nuclei rising and migrating toward the center of the tubules. Leydig cell-like nuclei are present. x 516.

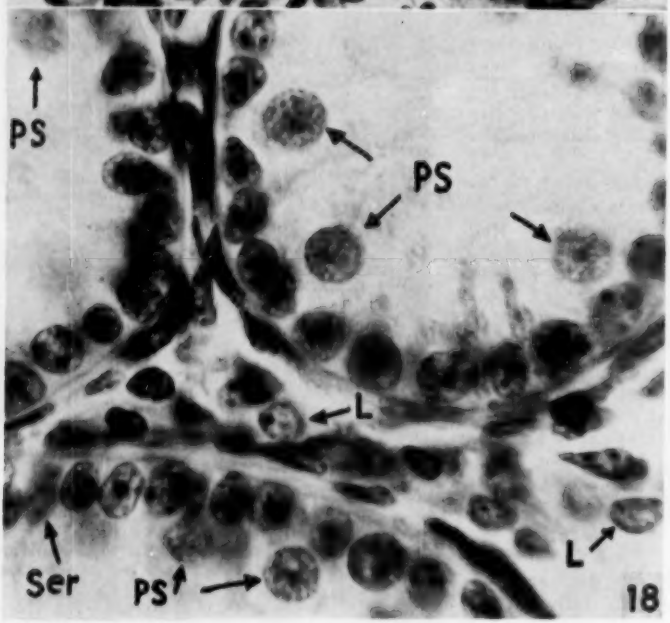


Fig. 18—Section from the same testis as in figure 16. This section of three seminiferous tubules shows nuclei of spermatogonia near the basement membrane, Sertoli cells (Ser, arrow), and primary spermatocytes (P S, arrow). The interstitial tissue shows the transition from mesenchymal cells to Leydig-like cells (L, arrow).

Hematoxylin-eosin stain. Section, 5 μ. x 990.

tubules appeared to have lumens. Some primary spermatocytes which arose from the basal cells were observed toward the center of the tubule where they were undergoing degeneration (fig. 20). Many of the Sertoli cells had disappeared.

At 90 days of age, the average diameter of the seminiferous tubules was 62.3 μ,

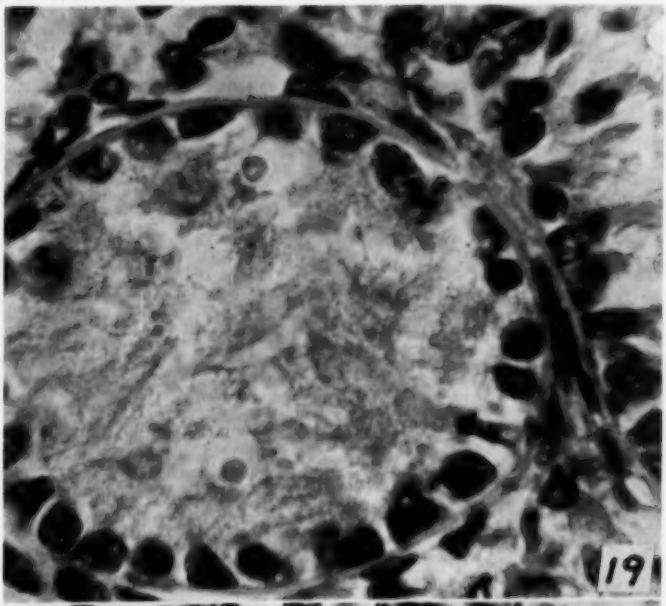


Fig. 19—Biopsy specimen of Jersey calf at 64 days of age. This photomicrograph shows the transversal and longitudinal section of two seminiferous tubules.

More than half the total number of cells near the basal membrane are in the stage of degeneration. Among them some spermatogonia begin to differentiate; differentiated Sertoli cells are present.

Hematoxylin-eosin stain. Section, 5 μ. x 990.

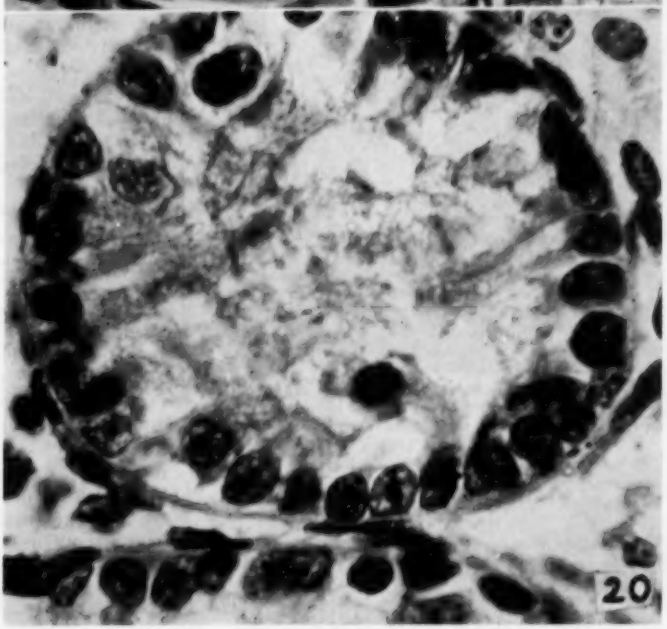


Fig. 20—Biopsy specimen obtained from the same Jersey calf as in figure 19 at 80 days of age. Transversal and longitudinal section of two seminiferous tubules. Most of the cells in the tubules are differentiated spermatogonia. There are far less degenerated cells on the wall of the tubules. The tubules are somewhat vacuolated.

Hematoxylin-eosin stain. Section, 5 μ. x 990.

and the average number of cells per tubule was 27. The only apparent difference from the 80-day biopsy was the fact that the intertubular spaces were smaller.

The fourth biopsy was made at 127 days of age. The diameters of the seminiferous tubules ranged from 63.0 to 120.0 μ—the average being 88.2 μ. The number of cells had increased until there was an average of 31 per tubule. The intertubular spaces were reduced in size and different stages of metamorphosis of the intertubular cells were observed—mesenchymal cells, fibroblastic cells, and those which resembled Leydig cells.

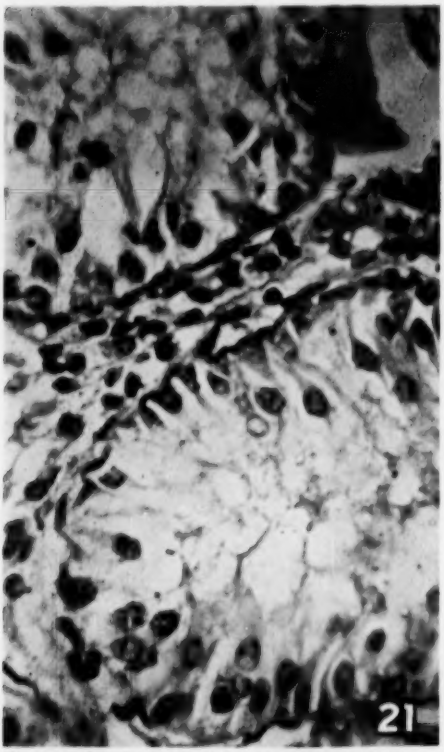


Fig. 21—Specimen obtained from the same Jersey calf as in figures 19 and 20; fifth biopsy at 127 days. This specimen was taken near the place of the previous biopsy.

The seminiferous tubules are vacuolated, and in the center along their axis there is almost complete liquefaction. The definitive germinal epithelium can be seen.

The presence of a large amount of Sertoli cells may be due to the fact that this specimen was taken near the place of the third biopsy or because Sertoli cells may be involved in the process of canalization of the tubules.

Hematoxylin-eosin stain. Section, 5 μ. x 516.

The seminiferous tubules continued to be vacuolated, and in the center along their axis there was almost complete liquefaction. The germinal epithelium presented two or three rows of cells. The large cells of these rows were primary spermatocytes, and a few of them had exfoliated into the center of the tubule (fig. 21). A large number of Sertoli cells was observed.

The animal was slaughtered at 168 days of age after having undergone five biopsies. The testes weight was 31.3 Gm., which was a little less than the weight given by Hooker [17] for animals of the same age. The diameter of the seminiferous tubules ranged from 120.0 to 153.0 μ. The tubule diameter appeared to be normal since Haq [14] reported that in the mature bull the diameter ranged from 175.0 to 340.0 μ. The average number of cells per tubule was 33.

The intertubular spaces were reduced a great deal and the connective tissue fibers were plentiful. Among the intertubular cells, the process of metamorphosis was more evident and Leydig cells appeared clearly defined. Almost all tubules contained fairly large lumens (fig. 22 and 23). Some primary spermatocytes which had previously exfoliated into the center of the tubules were now isolated in the lumens. There were not a large number of germinal cells. All cells had now differentiated into two generations of spermatogonia, primary spermatocytes, and a few spermatids.

DISCUSSION

This investigation shows that the normal development of the bovine testis, in both prenatal and postnatal animals, depends more upon the stage of development of individual animals than upon breed or age. This conclusion is based on the study of testicular weight, diameter of the seminiferous tubules, and the evolution of the histological elements of the testes of 21 animals.

As early as three months, the sex cords of a fetus exhibited two kinds of cells, the large and the small cells. Both histological elements continued to behave as different entities throughout prenatal development and for about two months of postnatal life. This observation of the existence of two types of cells in cattle is in agreement

with investigators who have studied other animals.

A point of controversy is whether the large cells, which are prominent in embryonic life, disappear without leaving any trace of their existence or whether they are primordial germ cells which give origin to the definitive germinal epithelium. Throughout this study, it was observed that in cattle the so-called primordial germ cells of the testis had nothing to do with the definitive germ cells. The primordial or large cells decreased gradually in number throughout the first month of post-

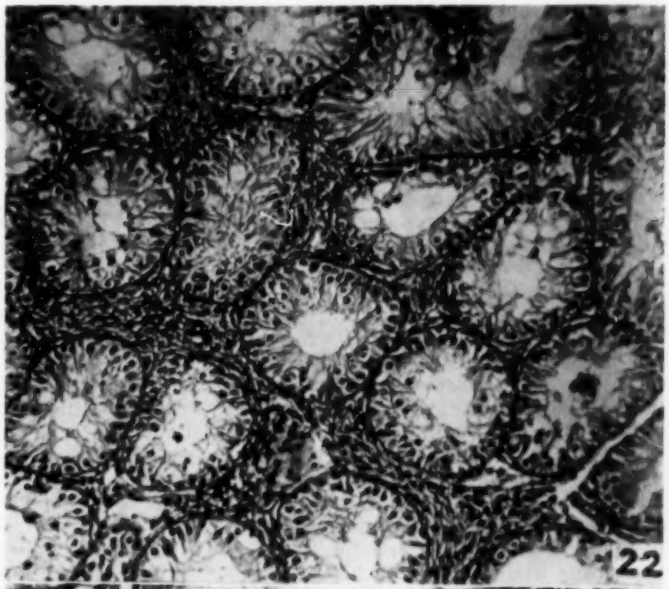


Fig. 22—Testis from the same Jersey calf as in figures 19 to 21; 168 days old. The intertubular spaces are reduced a great deal.

Among the intertubular cells the process of metamorphosis is more evident, and the Leydig cells appear more clearly defined. Some primary spermatocytes which had previously exfoliated into the center of the tubules are now isolated in the lumens.

Hematoxylin-eosin stain. Section, 5 μ. x 120.

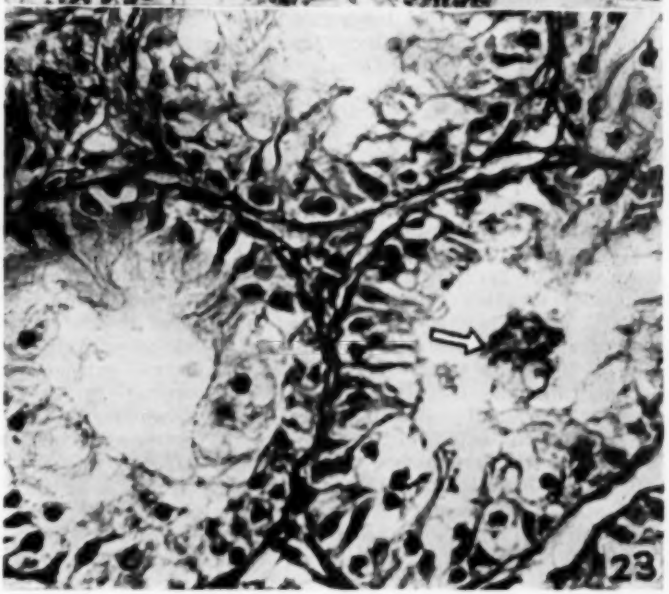


Fig. 23—Photomicrograph of the same section as in figure 22. The basement membrane, germinal epithelium, and interstitial tissue as shown here are regarded as typical of this age.

Debris of chromatin from the primordial cells and early primary spermatocytes still remain isolated in the center of the lumen (arrow).

Hematoxylin-eosin stain. Section, 5 μ. x 516.

natal life, and they had almost disappeared in animals of 70 days of age. On the other hand, the small epithelial cells which lay near the basement membrane and had a single type of nuclei began to grow. They increased to one, two, or even three times their original size; some remained attached to their parent cells and others migrated toward the center of the still solid tubules. Many of these cells were clearly differentiated primary spermatocytes. Among the cells observed in the center of the tubules some remained in the resting stage, others exhibited nuclear contraction, others degenerated at different stages of division, and still others gave rise to apparently normal cells which soon underwent degeneration. Later, these cells were reabsorbed with the tubular matrix in the process of formation of definite lumens of the seminiferous tubules.

In the calf testis, it was not difficult to trace the evolution of the large cells, since the various steps of development succeeded more slowly than in other animals which develop more precociously. It was rather easy to observe the different stages of this process in the longitudinal section of a seminiferous tubule. Furthermore, by the administration of thyroprotein, the seminiferous tubules became completely devoid of large cells and the cells of the basal membrane underwent a partial degeneration. Later, among the degenerated cells of the basal membrane, there arose two kinds of cells which were clearly distinguishable—the Sertoli cells, which were numerous, and the first generation of spermatogonia. Again, occasional cells, which arose from the basal layer, increased in size and moved toward the center of the tubules. At about 4 months of age when the seminiferous tubules began to form, that is, when the lumens appeared, the large cells of spermatogonial origin were exfoliated into the lumen and remained isolated until they were reabsorbed into the intratubular substance.

It is believed that this investigation demonstrates the fact that in cattle the definitive germinal epithelium arises from the indifferent cells which, according to Hargitt,[16] are descendents of the small cells of the genital ridge. However, as has been stated, the origin of the definitive germ cells has always been a point of controversy throughout embryological literature.

It is possible that the difference in results obtained by the various workers is due to the fact that some studied only isolated cases and, therefore, were unable to relate certain stages with others. Investigators, who carried out their studies in greater detail, agree on the point of degeneration and disappearance of the large cells.

Amitotic division was frequently observed in the fetal testis and continued to be present during the early development of the testis in postnatal life. In some preparations, large cells appeared which exhibited four nuclei, the explanation of which was found when another cell was observed at the last stage of amitotic division. This cell showed the nucleus about to end the complete separation but, before finishing the first division, both daughter nuclei began a new division.

Amitotic division, infrequently found in animals, was reported in the bull by Masui.[22] He found this division to occur in the penultimate spermatogonia but, according to his drawing, he showed that the large cells originated from the penultimate spermatogonia. His description of amitotic division in the spermatogonia must, therefore, correspond to that observed in large cells throughout this investigation.

In almost all preparations, giant cells were observed in some of the seminiferous tubules. These cells apparently contained the tetraploid number of chromosomes, but they were smaller than the normal ones. These cells in the bull testis appear to be similar to those reported by Painter[26] in man and Ranquini[27] in the horse. Although they appeared normal, the giant cells always represented a sign of degeneration.

More interstitial tissue was observed in the fetal testis than in that of the newborn and young animals. This fact has been interpreted, by Nelson[23] and others, to mean that the interstitial cells have something to do with the neutralization of the maternal estrogen or perhaps react in response to the chorionic gonadotrophic hormone. Because interstitial tissue disappears after birth and reappears about the time of puberty, it is believed that there are two distinct generations of Leydig cells. In the bovine testis of about 4 weeks of age, the intertubular spaces were filled with mesenchymal tissue; for this reason, Bascom[3] and Hooker[17,18] believed that

the Leydig cells originated from the mesenchymal tissue. Although repeated biopsies have been reported to affect spermatogenesis, the development and histological characteristics of the testis of the experimental bull appear to be no different from that of the normal testis, as reported by Hooker,[17] Haq,[14] and others. Apparently, the immature bovine testis is less sensitive to injury than that of adult animals. Histochemical studies, similar to those made by Cavazos,[7] will give valuable additional information to this work.

SUMMARY

1) The testes of 5 prenatal and 16 postnatal calves were studied in this investigation.

2) The testicular weight, diameter of the seminiferous tubules, and evolution of the histological elements of the testes of these 21 animals showed that the normal development of the bovine testis, both in prenatal and postnatal animals, depends more on the stage of development of the individual animals than on breed.

3) From early fetal life to about 4 months of age, the seminiferous tubules were filled with intratubular matrix. From this age to about 5½ months, the enlargement of the tubules and the dissolution of the matrix formed the definitive lumens.

4) The sex cords of a fetus of 3 months already exhibited two kinds of cells, the large and small cells, also called primordial and indifferent cells by many authors. These two kinds of cells continued to behave as different entities throughout fetal development to about 2 months of postnatal life.

5) The so-called primordial germ cells of the bovine testis gradually disappeared from the seminiferous tubules, leaving no trace of descendants.

6) The definitive germinal epithelium in the bovine testis arose from the small cells, which were lined along the basement membrane of the tubules.

7) Primary spermatocytes were observed in the testes of animals 70 and 71 days of age.

8) Amitotic division within the seminiferous tubules was frequent in prenatal and early postnatal life.

9) Giant cells were observed in many of the testes studied.

10) Testicular biopsy in immature bulls showed no noticeable effect on the normal development of the germinal epithelium.

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Relationship of Age to Susceptibility of Nonvaccinated Cattle to *Brucella Abortus*

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Most of the present knowledge concerning the relationship of age and the susceptibility of nonvaccinated cattle to *Brucella abortus* consists of accumulation of data obtained from numerous types of experiments and field studies, none of which were designed to provide specific information on the subject. Consequently, differences of opinion on that relationship have developed. Misinterpretations will be made if full consideration is not given to the effect of variations in dosage, methods, and virulence of *Br. abortus* challenge and of differences in herd management. This is emphasized by the facts that incidence and degree of infection are closely related to dosage, method, and virulence of exposure, and that heifers having had little or no association with infection prior to being bred expectedly would be more susceptible than adults that may have acquired some degree of immunity from subinfective exposure.

This study was designed to develop specific information on the relationship of age to susceptibility of nonvaccinated cattle to virulent *Br. abortus* and, thereby, to provide a basis for more accurately interpreting responses of cattle when exposed.

Materials and Methods

In this experiment, dairy cattle were used; 80 from the Brucella-free herd located at the Animal Disease Station, Beltsville, Md., and 6 purchased from three herds showing no evidence of brucellosis and in which vaccination was not practiced.

The cows ranged in age from 2 to 9 years. Of the 6 purchased cows, 1 was 5, 3 were 6, and 2 were 7 years old. All but 4 were pregnant from five to 190 days (average, 127 days) at the time of exposure to virulent *Br. abortus*.

Prior to exposure, cattle were grouped according to age and placed in experimental barns provided with either stall or stanchion isolation. In the stanchion-type barns, the cows were placed in alternate stanchions with a bar divider at each stanchion to prevent contact between animals. Several pre-exposure seroagglutinin titers were determined, using the standard AB8 tube and plate antigens and, at the same time, blood was collected for bacteriological examination. The methods employed were those described by Manthei and Carter,[1] except the blood-broth mixture was plated on a solid medium after seven to ten days of incubation. No evidence of Brucella infection was detected in any of the animals.

The susceptibility of the cattle to brucellosis was determined by artificial exposure to the highly virulent *Br. abortus* strain 2308. The methods of preparation of exposure material and its instillation on the conjunctiva were those described by Manthei *et al.*[2]

All cattle were exposed on the same day to approximately 662,600 viable cells of Brucella strain 2308; the concentration was confirmed by actual plate counts immediately before and after exposure of the animals in each barn.

Subsequent to exposure, seroagglutinin titers were determined at weekly intervals for 32 weeks, then at varying intervals until the animals were disposed of.

At the time of parturition, uterine contents, secretions from each quarter of the udder, blood, and, in the cases of abortion, five organs and six portions of the digestive tract from each fetus were collected and cultured for Brucella. All specimens except blood were cultured on potato-infusion agar containing 7.5 per cent horse blood serum in an atmosphere of 10.0 per cent CO_2 at 37 C. for seven days. Each quarter sample of colostrum was examined for Brucella agglutinins and also injected intraperitoneally in 5.0-ml. amounts into each of 2 guinea pigs. Uterine contents were cultured in the same manner as the colostrum, and each of 4 guinea pigs were inoculated with 0.5 ml. subcutaneously. Guinea pigs were killed and examined for gross lesions approximately five weeks after inoculation. Blood samples were collected from the guinea pigs for agglutinin titer determinations, and spleens were removed for bacteriological examination. In cases where cows showed evidence of being infected but had udder secretions that were negative for Brucella at the time of parturition, additional samples of these secretions were subsequently collected at weekly intervals and examined by the methods previously mentioned.

All bacterial isolations suspected of being Brucella were identified and typed in accordance with procedures recommended by WHO and FAO Expert Committee on Brucellosis.

Smears were made of chorionic (fetal) cotyledons from some of the cows and stained according

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TABLE 1--Response of Various Age Groups of Nonvaccinated Cattle Exposed to Virulent Brucella Abortus

Age groups	No. of cattle	No. pregnant	No. of premature parturitions	No. with uterine infection	No. with udder infection	Total No. Brucella positive	No. with persistent titer
2 years	21	21	14	15	16	17	17
3 years	18	18	15	16	14	16	16
4 years	17	17	11	11	13	13	13
5 years	11	9	5	6	5	7	6
6 years	10	10	5	6	7	7	7
7 or more years	9	7	4	5	7	7	8

to the method described by Christoffersen and Ottosen,[3] a modification of the Køster staining procedure described by Hansen and Køster.[4]

Two methods of evaluating results of this experiment were applied to the data concerning responses of individual cows and of various age groups of cows for determining their susceptibility to virulent *Br. abortus*. One of these is based on percentage of infection, the procedure used most extensively in the past. Because this method has not clearly defined the various degrees of infection, a method of numerically evaluating responses, the one introduced and discussed at the annual meeting of the Brucellosis Research Conference in 1954, was also used. However, since a majority of agreement had not been attained on the numerical values suggested for each response, values were assigned by the authors which appeared to provide a concise method that would accurately measure the degree of infection.

Credit for the introduction and major development of this method is given to the members of the Brucellosis Research Conference.

The numerical evaluation of responses of cattle to virulent *Br. abortus* is as follows:

Response	*Value*
1) Pregnancy terminating with abortion or premature calf and isolation of Brucella from uterine contents	5
2) Pregnancy terminating with full-term calf and isolation of Brucella from uterine contents	4
3) Isolation of Brucella from udder secretion	3
4) Two or more dilution increase of postexposure seroagglutinin titer that persists for 90 days or longer	2

By using the numerical evaluation procedure, the degree of infection in an individual may vary from a minimum with a value of 2 to a maximum with a value of 10. An animal with the maximum degree of infection is one that aborted and had uterine and udder infections and showed a two or more dilution increase in seroagglutinin titer that persisted for 90 days or longer.

RESULTS

The response of the six different age groups of nonvaccinated cattle to virulent *Br. abortus* is shown (table 1). Although there were some differences in the clinical response between age groups, the total evidence of infection was not significantly different. Furthermore, the slight difference in total response between age groups was not significantly greater than that between cattle of each age group. Since the majority of animals that aborted had udder and uterine infections and an increase of two or more dilutions in seroagglutinin titers which persisted for at least 90 days, only those animals showing a different response in each of the following groups will be discussed:

Two years of age—there was no isolation of *Br. abortus* or serological evidence of infection in 1 of 14 animals that aborted. Of the 4 infected animals that gave birth to full-term calves, 2 had uterine infection and 3 had udder infection.

Three years of age—*Br. abortus* was isolated from the uterine material of 15 cattle that aborted; however, only 13 had udder infection. In addition, both uterine and udder infections were demonstrated in 1 animal that gave birth to a full-term calf.

Four years of age—2 animals that gave birth to full-term calves had only udder infection.

Five years of age—2 cows, 1 that aborted and 1 that gave birth to a full-term calf, had only uterine infection. Only udder infection was demonstrated in 1 of 2 nonpregnant animals.

Six years of age—of the 5 cows that had full-term calves, *Br. abortus* was isolated from the udder secretions of 2, and 1 of these had uterine infection also.

Seven or more years of age—1 of the 3 cows that had full-term calves had udder and uterine infections and 1 had udder infection only. In addition, *Br. abortus* was isolated from the udder secretions of 1 of the 2 nonpregnant cows.

Use of an increase of two or more dilutions in postexposure seroagglutinin titers

TABLE 2—Degree of Infection in Various Age Groups of Nonvaccinated Pregnant Cattle Exposed to Virulent Brucella Abortus

Age groups	No. of cattle	Brucella positive (%)	Index of infection	Completely susceptible (%)	Partially resistant (%)	Completely resistant (%)
2 years	21	80.9	7.9	61.9	19.0	19.0
3 years	18	88.9	8.5	72.2	16.7	11.1
4 years	17	76.5	7.1	64.7	11.8	23.5
5 years	9	66.7	5.7	44.5	22.2	33.3
6 years	10	70.0	6.4	50)	20.0	30.0
7 or more years	7	85.7	7.7	57.1	28.6	14.3

that persisted for at least 90 days was an efficient method of determining the brucellosis status of cattle. This method correctly identified 66 of the 67 infected animals and 18 of the 19 noninfected animals. One of the two discrepancies occurred in a 5-year-old cow that had uterine infection at the time of giving birth to a full-term calf, and the other one in a 7-year-old noninfected, nonpregnant cow. Brucella was not isolated from either cow at the time of necropsy, which was approximately eight and one-half months following exposure.

The lower incidence of abortion in each of the last three age groups was directly associated with purchased cattle. Although the 6 purchased cattle showed no Brucella agglutinins in the 1:25 dilution of their serum prior to exposure, only 1 of 6 aborted as compared with 13 of 20 which were raised in the Brucella-free unit at the Animal Disease Station. Furthermore, only 2 of 6 purchased cows became infected as compared with 17 of 20 raised at Beltsville.

Brucella organisms were demonstrated by the Køster staining method in the smears of cotyledons from 28 of 29 cattle that had uterine infection, whereas none were observed in the smears from 6 noninfected cattle. This limited use of the Køster staining method further suggests its value as a means of rapidly demonstrating uterine infection in cattle suspected of having brucellosis.

Application of the proposed method of evaluating the response of cattle to virulent *Br. abortus* and comparison of it to percentage of infection which has been used in the past are presented (table 2). Because of the criterion employed in the proposed method of evaluation, it can be applied only to pregnant cattle. The figures presented under the column "index of infection" are the arithmetical means of the sum of the numerical values that represent the responses of cattle in each age group. Although the percentage of infection has a tendency to parallel the index of infection in most age groups, it does not reveal the degree of infection that is expressed by the the latter. The information presented in the last three columns of this table further illustrates the desirability of using the index of infection for measuring degree of resistance or susceptibility.

Discussion

The data presented clearly demonstrated that there was no significant difference in susceptibility of nonvaccinated cattle of different ages to virulent *Br. abortus* when the animals were raised in a Brucella-free environment. This information provides more accurate standards for evaluating results of various aspects of immunological studies on vaccinated cattle. In addition, it greatly increases the flexibility of effective usage of cattle of different ages in brucellosis research.

The difference in both abortion and infection rates between animals raised under carefully controlled conditions and those selectively purchased was highly significant. This is not the first time differences in susceptibility between these two types of cattle have been observed, but it again emphasizes the paramount necessity of raising experimental animals of known origin under carefully controlled environmental conditions if accurate interpretations are to be made of research results.

Employment of the index of infection more clearly illustrates the degree of resistance or susceptibility than the percentage of infection which has been used for this purpose in the past. When this index is applied to past and present research results, it appears to be a reliable method of evaluating the response of vaccinated and nonvaccinated cattle challenged with virulent *Br. abortus*.

Although use of the two or more dilution increase in seroagglutinin titers proved to be an effective method of determining the brucellosis status of cattle under controlled experimental conditions, the procedure obviously has no application as a diagnostic aid in field studies or in a control program, since it is impossible to determine when an animal receives exposure under these conditions.

SUMMARY

There was no relationship of age to susceptibility of nonvaccinated cattle to virulent *Brucella abortus*.

When exposed to virulent *Br. abortus*, the susceptibility of cattle purchased from herds showing no evidence of brucellosis was demonstrated to be significantly less than that of cattle raised in a carefully controlled Brucella-free environment.

Application was made of a numerical index of infection which more clearly illustrated the degree of susceptibility of nonvaccinated cattle to virulent *Br. abortus* than did the percentage of infection. Furthermore, this index has been applied to unpublished results of vaccination studies and was effective in measuring the degree of immunity of cattle to brucellosis.

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Pathology of a Bovine Mucosal-Type Disease

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In recent years, considerable attention has been directed to a newly recognized group of bovine diseases characterized primarily by diarrhea and erosions of the gastrointestinal tract. Olafson *et al.*[17] Ramsey,[19] Pritchard *et al.*,[18] and Hollister *et al.*[12] reported various disease entities belonging to this group, and Miller [14] and others reported on an anterior respiratory syndrome group. Although these various conditions appear to be specific entities, a number of clinical and pathological features are held in common.

Recently, another variant of the mucosal disease type was observed in Virginia. A preliminary report on the clinical findings, gross pathology, and transmission studies has been published.[11] The purpose of the present paper is to describe the pathological findings in this variant and to compare these findings with the published observations on other members of the mucosal disease complex. Necropsy and histopathological findings of 14 natural cases and 6 experimental cases in calves form the basis of this report.

Clinical Findings

The disease occurred in two widely separated commercial beef herds. In both instances, morbidity was restricted to weanling animals 6 to 9 months old. Adult animals and young calves in direct contact with the affected group never showed evidence of disease. The clinical course was characterized by high fever, fetid mucoid diarrhea, dehydration, lameness, and mild lacrimation and salivation. Pneumonia was a frequent, late complication. Morbidity approached 100 per cent in the affected group, while mortality averaged 50 per cent. Animals died two to four weeks after onset or recovered within four to eight weeks.

Transmission of the disease to young dairy-type calves was accomplished by contact exposure and by intramuscular inoculation of Seitz-filtered blood or spleen emulsion from moribund animals. The experimental disease was mild with a diphasic temperature rise and transient neutrophil leukopenia. Buccal erosions, anorexia, and transient diarrhea were usually observed. Recovery was rapid.

Necropsy Findings

Natural cases of the disease showed dehydration, dry lusterless eyes, nasal discharge, and plastering of the tail and hindquarters with fetid, mucoid fecal material. Erosions occurred in the interdigital space of one or more feet of all animals. They varied from minute, linear, ragged-edged, brownish elevations to multiple coalescing erosions or ulcerations (fig. 1). Secondary, necrotizing infection was prominent in the more extensive ulcerations.

Similar lesions, varying from minute (1 to 2 mm.) brownish elevations or erosions to necrotic ulcers 1 cm. or more in diameter, were uniformly observed on the dental pad, hard and soft palate, inside the upper and lower lips, behind the corner incisors, oral surface of the cheeks, sides of the tongue, and the floor of the pharynx. No lesions were found on the dorsum of the tongue. There was considerable individual variation in the number, location, and severity of the oral lesions. In general, they tended to be scattered and rarely coalesced. Occasionally, a small, fine erosion was present in the external nares or rostral third of the turbinate mucosa.

Secondary necrotizing infection of the oral lesions was usually accompanied by enlarged, purulent suprapharyngeal lymph nodes. Rarely, a necrotic ulcer was present in the dorsal commissure of the vestibule of the larynx. No lesions were observed in the trachea.

Multiple fine, linear, brownish superficial lesions were regularly observed in the caudal third of the esophagus and only rarely in the cranial two thirds (fig. 2).

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The author is indebted to Mrs. Eugene Manus for preparing the sections and to Dr. Warren G. Hoag for his continued assistance and consultation. Dr. J. E. T. Laningham assisted with a number of the necropsies.

The early lesion was a brownish, linear elevation of the mucosa which progressed to superficial, ragged-edged, coalescing erosions. Secondary infection and ulceration were less common than in the interdigital and buccal lesions.

Pneumonia, present in 70 per cent of the natural cases, involved the ventral portions of the apical and cardiac lobes of both lungs. Dry, purplish red consolidation, purulent exudate in the bronchi, and interstitial edema characterized the pneumonia. The bronchial and mediastinal lymph nodes were edematous and reddened in animals with severe pneumonia.

Erosions and ulcerations were found in the esophageal groove and sulcus omasi of a few animals. Petechial hyperemia, submucosal edema, and shallow erosions were frequently observed in the fundic folds of the abomasum. Ragged or smooth-edged hemorrhagic ulcers were invariably present in the pyloric mucosa.

The contents of the small intestine, cecum, colon, and rectum were of a watery, fetid, mucoid nature. Only scattered areas of hyperemic mucosa were encountered in the small intestine. Gross lesions were not observed in the Peyer's patches. The cecum and proximal loop of the ascending colon were diffusely reddened. The mucosa of the coiled loop and distal loop of the ascending colon, descending colon, and rectum was commonly flecked with freckle-like, petechial hyperemia. The Peyer's patches at the ileocecal valve and at the junction of the proximal and coiled loops of the ascending colon were usually reddened with dilatation and plugging of gland openings with creamy mucopus.

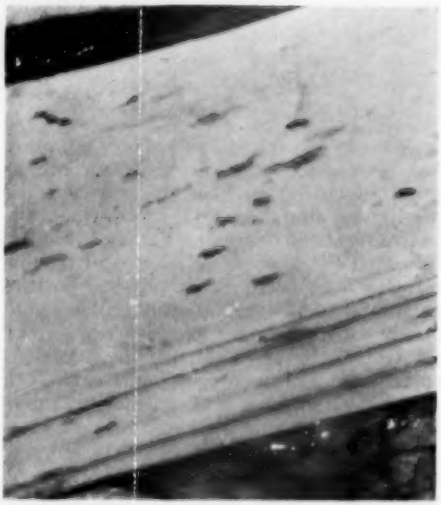

Fig. 2—Enlarged photograph of linear erosions in the caudal third of a bovine esophagus.

Liver lesions, present in all cases, varied from small, focal, yellow-white areas of necrosis to diffuse, severe fatty degeneration. The gallbladder contained thick, viscid, flocculent bile but never showed mural lesions. The kidneys presented subcapsular petechiae; swollen, pale, flabby paren-

Fig. 1—Erosions in the interdigital skin of a bovine foot.

Fig. 3—Early ballooning and nuclear pyknosis of cells in the outer third of the stratum spinosum (esophagus). Gömöri stain. x 240.

chyma; and corticomedullary reddening in 90 per cent of the cases.

Experimental calves developed mucosal lesions qualitatively identical to those in field cases in both nature and distribution. The lesions were not as prominent in the calves, never progressing to the extensive, necrotizing ulcerations common in the natural cases. Pneumonic and renal lesions were not observed, but minimal white foci were usually present in the livers of the calves.

Histopathology

The lesions in stratified epithelium (interdigital skin, mouth, esophagus, esophageal groove) showed considerable gross and microscopic variation apparently related to their extent, degree of secondary infection, and age. The fundamental pathogenesis of all the lesions, however, appeared to be identical. The early, small, elevated lesion observed grossly was characterized microscopically by focal areas of ballooning of the cells in the outer third of the stratum spinosum (fig. 3). The ballooning was the result of either hydropic degeneration or vacuolation of the cytoplasm with accompanying nuclear karyolysis or, more commonly, pyknosis. As the cells swelled, the intercellular bridges disappeared, and narrowed intercellular areas filled with homogeneous acidophilic

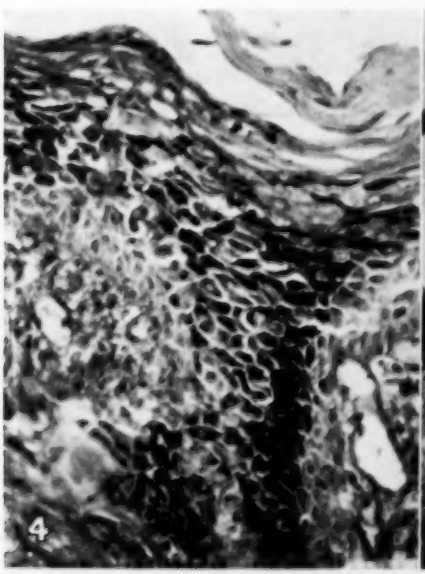

Fig. 4—Glycogen-rich cells in a rete peg. Early ballooning degeneration is apparent in the stratum spinosum (esophagus). Periodic Schiff stain. x 240.

Fig. 5—Marked ballooning of stratum spinosum with coalescence. Atrophic changes are apparent in the stratum cylindricum (arrow). Gömöri stain. x 150.

material. The inner two thirds of the stratum spinosum and the stratum cylindricum remained intact. Frequently, glycogen could be demonstrated in the epithelial cells of the spinosum and cylindricum subjacent to areas of ballooning degeneration (fig. 4). The significance of this observation will be discussed later. No lipid or Schiff-positive material could be demonstrated in the ballooned cells in carbowax preparations.

As the lesion progressed, the stratum corneum was first bulged then ruptured and frayed by the continued ballooning of the stratum spinosum. The fraying of the stratum corneum provided an explanation for the ragged appearance of the small gross lesions. Eventually, the entire spinosum was involved, and the cells of the stratum cylindricum showed regressive changes (fig. 5). The ballooning cells ruptured and coalesced forming vesicle-like areas containing nuclear remnants, filmy strands of cytoplasmic debris, and occasional neutrophils. Inflammatory reaction was minimal, with some capillary dilatation in the dermal papillae and a few, scattered neutrophils.

Finally, the necrotic material sloughed away and a shallow erosion remained. Healing could apparently occur at this stage with regrowth of epithelium from

Fig. 6—A healing erosion showing separation of necrotic debris (arrow) and epithelial hyperplasia. There is mild fibroplasia in the dermis. Hematoxylin and eosin stain. x 32.

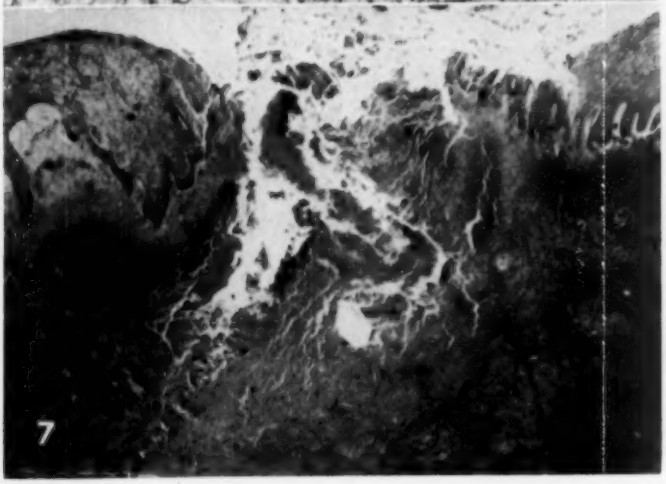

Fig. 7—A necrotic ulcer with ragged edges showing ballooning degeneration (buccal mucosa). Gömöri stain. x 32.

the edges of the erosion and hyperplasia of the rete pegs adjacent to the damaged area (fig. 6). Usually, the stratum cylindricum was destroyed, secondary bacterial invasion occurred, and a necrotic ulcer, extending into the underlying connective tissue, developed (fig. 7). A marked neutrophil, lymphocyte, and fibroplastic reaction developed around the expanding base of these necrotic ulcers. Hyperplasia of the epithelial rete pegs commonly occurred at the margins of the ulcer.

No damage was detected in the adnexal glands except when involved by deep, necrotic ulcerations.

The rare erosions in the turbinate mucosa were characterized by necrosis and sloughing of the pseudostratified epithelium with minimal inflammatory reaction (fig. 8).

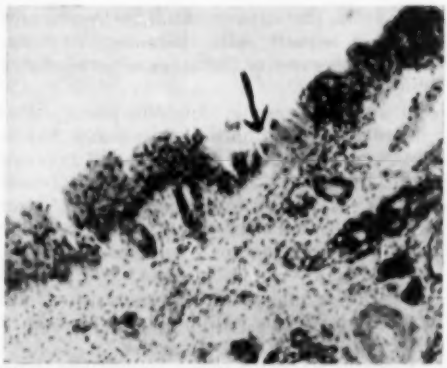

Fig. 8—Erosion in nasal turbinate mucosa. Normal pseudostratified epithelium is to the left, necrotic cells in center (arrow), and complete denudation to the right. Gömöri stain. x 120.

The histological lesions in the abomasum, small intestine, and colon tended to be mild, scattered, and focal in nature. The fundus of the abomasum frequently showed diffuse submucosal edema and hyperemia (fig. 9). Dilated, hyperemic capillaries and focal hemorrhages in the lamina propria corresponded to the petechiae observed grossly. Edema and hyperemia tended to be more localized in the pyloric portion of the abomasum. In both regions focal epithelial necrosis occurred, leading to grossly visible erosions. The erosion appeared to be preceded by a remarkable coagulation necrosis which began superficially and extended toward the muscularis mucosae, destroying all the epithelial elements of the glands. (The parietal cells of the fundic glands were somewhat more resistant than the neck or chief cells.) The connective tissue framework of the lamina propria persisted briefly, then collapsed, and disappeared (fig. 9). Capillary dilatation, edema, and hemorrhage were seen in the lamina propria in these areas. Inflammatory cell infiltration was minimal unless secondary bacterial invasion occurred.

Less commonly, a more selective necrosis of the epithelium lining individual glands was seen. The epithelial cells became atrophic and necrotic and sloughed into the glandular lumen. Eventually, the lumen dilated and filled with mucus, desquamated epithelial cells and neutrophils. Atrophic, flattened epithelial cells lined these crypt abscesses (fig. 10). The abscesses usually occurred singly and never appeared to be present in sufficient numbers to produce gross lesions.

Submucosal and lamina propria hyperemia and edema were present in only scattered areas of the small intestine. Crypt abscesses identical to those described in the abomasum were frequently found in the hyperemic areas but never in sufficient numbers to produce gross lesions. Significant lymphorrhexis was observed in only one Peyer's patch in 1 animal.

Single, scattered crypt abscesses, identical to those in the small intestine, were present in relation to focal areas of hyperemia and hemorrhage in the lamina propria of the cecum and colon (fig. 10). Crypt abscesses were prominent grossly and microscopically in relation to the Peyer's patches at the ileocecal valve and at the junction of the proximal and coiled loops of the ascending colon (fig. 11). The submucosal glands in these areas, particularly, were dilated, filled with mucus, cellular debris, and neutrophils, and lined by atrophic, flattened epithelium.

The liver presented gross fatty degeneration in all but 1 of the natural cases and minimal focal necrosis in the experimental calves. The distribution and location of the fat was irregular from lobule to lobule and from section to section (fig. 12). The distribution of fat was diffuse and severe but presented no specific periportal or centrilobular predilection. Multiple, small cytoplasmic droplets coalesced to form a single large vacuole which displaced and compressed the hepatic cell nucleus. In some subcapsular foci, fatty cysts devel-

oped from the rupture and coalescence of fat-laden hepatic cells. Bile capillary stasis was observed in the more severely fatty lobules.

Minimal, variable lymphocytic portal infiltrations were noticed. In many livers, scattered microfoci of hepatic cell necrosis and Kupffer cell hypertrophy were found in both fatty and nonfatty lobules. A few neutrophils were often present in these foci. The focal necrosis was the only finding in 1 field case and in the experimental calves. No microscopic lesions were encountered in the gallbladder and biliary tract.

Kidneys showing gross lesions histologically presented the picture of anoxic or so-called lower nephron nephrosis.[3] Scattered microfoci of perivascular, interstitial infiltrations of lymphocytes, and small areas of fibrosis were present in the cortex. There was variably severe hyperemia and hemorrhage at the corticomedullary junction together with marked cytoplasmic vacuolation and swelling of the epithelium of the Henle loops. No lipid or Schiff-positive material could be demonstrated in these vacuoles. Swelling, granulation, and chromatolysis were less commonly observed in the convoluted tubules. Granular and cellular casts were frequently present in the collecting tubules of the medulla.

The spleen showed no gross lesions and presented a highly variable histological appearance. There was usually evidence of lymphocyte depletion and perifollicular

Fig. 9—Fundic fold of abomasum. Submucosal edema and hyperemia are prominent. Normal glandular epithelium is at right. There is virtually complete epithelial coagulation necrosis on the left with persistence of the skeleton of the lamina propria. Hematoxylin and eosin stain. x 32.

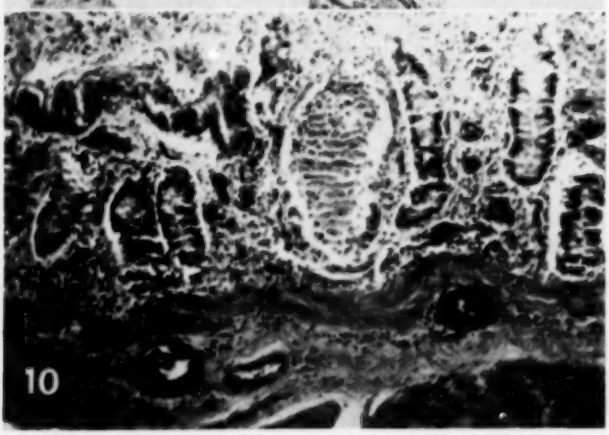

Fig. 10—Crypt abscess in colon. There is submucosal and lamina propria hyperemia. Gömöri stain. x 120.

hyperemia. Megakaryocytes, neutrophils, and hemosiderophages were present in moderate numbers.

Only lymph nodes draining necrotic, suppurative areas contained significant lesions. Reaction centers, edema, and neutrophil packing of the capsular and medullary sinuses were seen in the suprapharyngeal, bronchial, and mediastinal lymph nodes. The thymus was identified as a remnant in 1 animal and, microscopically, was severely hyperemic, hemorrhagic, and virtually devoid of thymocytes.

No lesions were found in the adrenal glands. An evaluation of lipid vacuolation in the cells of the zona fasciculata showed all possible variations from no demonstrable vacuoles to marked, widespread vacuolation. The fasciculata of the bovine adrenal normally shows few or no lipid droplets.[16]

Pneumonia was of the fibrinous, bronchopneumonia or, most commonly, mixed type. The typical picture was one of peribronchiolar and intraluminal accumulations of lymphocytes and neutrophils with bronchiolar epithelial necrosis and sloughing. Alveoli filled with edema fluid, fibrin, and neutrophils were interspersed with areas of atelectasis and alveolar foam cell accumulations. Interlobular septal edema was prominent in a number of lungs.

No gross or histological lesions were encountered in the brain, pituitary, thyroid, salivary glands, pancreas, heart, vessels, skeletal muscle, urinary bladder, genitalia, or peripheral nerves that were examined. The bones and joints were examined grossly and showed no lesions.

Discussion

The changes described in the stratified epithelium can not be considered pathognomonic of this disease. They do differ, however, from the lesions found in several other bovine epithelial diseases. In the present case, the essential lesion appears to be hydropic, ballooning degeneration of epithelial cells in the outer third of the stratum spinosum. Simultaneously, glycogen may accumulate in the cylindricum and nonhydropic spinosum epithelial cells. Montagna[15] describes the occurrence of glycogen in basal and spinosum cells in injured human epidermis. Glycogen accumulation may indicate early cellular

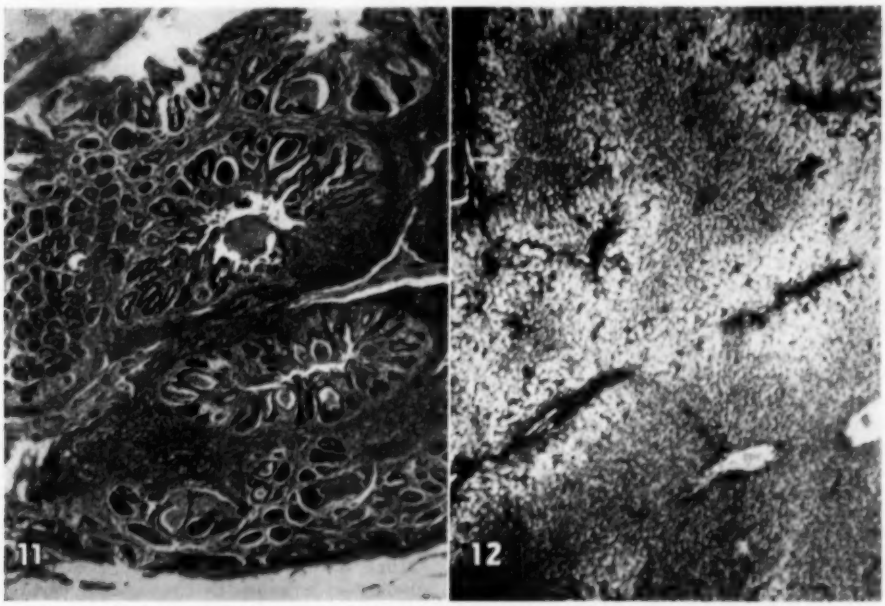

Fig. 11—Multiple crypt abscesses in submucosal glands at junction of proximal and coiled loops of ascending colon. Hematoxylin and eosin stain. x 32.

Fig. 12—Fatty degeneration of liver with heavier periportal infiltration. Gömöri stain. x 32.

damage manifested as an abnormality in the glycolytic system. Finally, the entire spinosum becomes hydropic, the stratum corneum bulged and ruptured, and erosion occurs.

In rinderpest, the necrosis begins in the deep part of the stratum spinosum and is characterized by pyknosis and shrinkage rather than ballooning of affected cells. Also, in rinderpest, the margins of the erosions are sharply demarcated and bounded by essentially normal epithelium.[12] The erosions in the present disease are ragged and the ballooning degeneration extends a variable distance from the ulcer margins

The primary lesion in vesicular stomatitis appears as a spongiosis in the stratum spinosum with loss of intercellular bridges, cellular dissociation, and intercellular vacuolation.[4, 5] Chow *et al.*[4] mention "an early stage of ballooning degeneration of the epidermal cells" in the interdigital skin at one site in 1 animal.

Frenkel [9] described the epithelial lesion in foot-and-mouth disease as a degeneration and shrinkage of cells in the stratum spinosum with spongiosis and vesicle formation.

Early pox lesions rather closely resemble the lesions in the present disease.[2, 3] However, the inclusion bodies, gross vesiculation, and pustulation characteristic of pox have not been observed in this disease.

The focal coagulation necrosis in the abomasum apparently represents a nonspecific reaction to diverse forms of injury. Similar lesions have been observed in normal calves, slaughter animals, and in animals with a variety of disease conditions.

The crypt abscesses in the abomasum, small intestine, and colon may represent a specific virucidal effect on the glandular epithelium. Maurer *et al.*[13] felt that the rinderpest virus invades the intestinal epithelium. Seibold,[21] describing Ramsey-type mucosal disease, believes the essential alteration is damage to the glandular epithelium. Ramsey [19] considers acute catarrhal enteritis the initial lesion apparently followed by distension of the glands and pressure atrophy of the epithelium.

Follis [8] discussed similar changes in pigs with pantothenic acid deficiency and in human pellagrins. Dunne *et al.*[6] described the lesion in pigs recovering from niacin deficiency. The available evidence appears to indicate that crypt abscess formation is specific for the enteric glandular structure and not for any particular disease.[20] The basic pathogenesis of the lesion remains to be worked out. The same generally holds true for the cystic submucosal glands described [20] in the Peyer's patches at the ileocecal valve and junction area of the colon.

The fatty degenerative changes in the livers apparently accompanied the anorexia and cachexia characteristic of this disease. The focal necrosis may be a specific lesion, a result of anoxia, or simply a reaction to severely fatty hepatic cells. The interstitial nephritis, Henle loop vacuolation, and corticomedullary hyperemia agree with Boyd's description of anoxic nephrosis in man.[3] Both the hepatic and renal lesions probably represent secondary phenomena, but are of considerable interest and importance in understanding the pathological physiology of the disease. The failure of antibiotic and sulfonamide therapy to alter the course of the disease may be at least partially understood when the renal and hepatic lesions are considered. The nephrosis, particularly, was often severe enough to indicate that uremia is a contributing cause of death.

Essential histopathological differences between the present disease, foot-and-mouth disease, rinderpest, vesicular stomatitis, and pox diseases have already been discussed. The rhinotracheitis described by Miller [14] affects primarily the respiratory tract, presenting entirely different gross lesions. Hollister *et al.*[12] have not presented histopathological findings in "muzzle disease" as yet, but some of the gross lesions appear to be different from those in the present disease.

Ramsey's mucosal disease [19] is pathologically similar to the present disease. The stratified epithelial lesion illustrated in Seibold's figure 2 [21] appears to be almost identical. Important differences seem to be the lower incidence of lameness (10 % as opposed to 100 % in our disease), more severe, coalescing buccal erosions, erosions on the dorsal surface of the tongue, and more extensive lesions in the small intestine and colon. The virus diarrhea disease described by Pritchard *et al.*[18] may be differentiated by the lameness resulting from laminitis rather than erosions and the greater frequency of erosions in the cranial third as opposed to the caudal third of the esophagus. The histopathological changes of that condition have been inadequately described

and can not be compared. Lameness is not described in the virus diarrhea of Olafson *et al.*[17] and its histopathological changes have not been described. Animals with malignant catarrhal fever show nervous symptoms and lesions of nonsuppurative viral encephalitis. Intracytoplasmic inclusion bodies have been described in the epithelial lesions.[10]

Flina and Jensen,[7] studying necrobacillosis of the bovine foot, reported that, "Epithelial cells of necrotic tissue and of tissue adjacent to the necrosis were enlarged and showed hydropic degeneration of cytoplasm." There is an interesting parallel between this description and the epithelial lesions in the present mucosal disease.

The earlier stages of ecthyma contagiosum in sheep, described by Wheeler and Cawley,[22] show ballooning degeneration similar to that described in this report. The marked granulomatous, papillomatous later stages have not been seen in the present disease.

SUMMARY

The pathology of a mucosal-type disease occurring as an epizootic in Virginia has been described. Primary lesions were localized in the epithelium of the gastrointestinal tract, interdigital skin and, rarely, the turbinate mucosa. Ballooning degeneration was a feature of the stratified epithelial lesions, while coagulation necrosis and crypt abscesses characterized lesions in the columnar epithelium. Secondary lesions in the lungs, livers, and kidneys were common and contributed significantly to mortality.

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A Survey of the Gastrointestinal Parasites of Cattle in North Carolina

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CATTLE NUMBERS in North Carolina have increased 43.5 per cent during the six-year period from 1949 to 1955. Acreage of improved pasture has not increased at the same rate but the carrying capacity of pastures has through improved management practices. More cattle per acre of pasture has intensified the parasite problem. Economics of production have forced more and more of the attention of farmers and animal disease workers to this problem. Before initiating a program for the control of parasites of cattle in North Carolina, it seemed desirable to determine the relative numbers of the various species of parasites in a given area by a survey. The purpose of this survey was to obtain as much information as possible on the numbers and species of gastrointestinal parasites in cattle in relation to the geographic areas of the state.

MATERIAL AND METHODS

Materials.—Material was collected from animals at the time of slaughter at slaughterhouses in 19 localities in North Carolina (fig. 1). Small local slaughter plants were selected in order to increase the probability that the animals were native cattle.

No selection of animals was made except to collect from adults, yearlings, and calves at each location. At some locations, it was not possible to get all three age groups. The age was determined by observation before and at slaughter.

Viscera from calves which were determined to be receiving milk were not examined. Most of the animals were in fair to good flesh. The rumen was not used in this survey; therefore, no information on the incidence of rumen parasites was obtained. Collection of material extended from March, 1955, to July, 1956.

Methods.—At the time of slaughter, the abomasum was dissected from the omasum and the omasal and pyloric openings were tied. The small intestine was tied at the pylorus and ileocecal junction. The large intestine was tied approximately 18 inches from the anus and the posterior portion discarded. The intestinal tracts were placed in cans and returned to the laboratory. At the laboratory, the tracts were dissected out, separated, and the abomasum, small intestine, and cecum and large intestine opened into separate containers and thoroughly washed in water. This material was run through a 60-mesh (per inch) screen. The material remaining in the screen was placed in a 1-gal. jar and sufficient formalin and water were added to a final concentration of 10 per cent formalin.

In examining these specimens, an aliquot was taken from the jar and small amounts placed in a Petri dish with concentric circles drawn on the bottom. A dissecting microscope was used to locate the worms. All the parasites in the aliquot were counted. A generic identification was made on the first 100 worms observed (or on all contained in the sample). No attempt was made to identify species. The tapeworms were counted and identified individually.

TABLE 1—Abomasal Parasites—Number Recovered by Age of Animal and Locality

Animal age	No. of animals	Haemonchus spp.	Ostertagia spp.	Trichostrongylus spp.
TOTALS FOR COASTAL PLAIN AREA				
Adult	24	457	3,607	935
Yearling	44	107	3,361	4,637
Calves	23	200	1,322	564
Average	91	223	2,925	2,631
TOTALS FOR PIEDMONT AREA				
Adult	35	33	448	123
Yearling	21	380	4,317	2,954
Calves	12	187	1,234	208
Average	68	167	1,781	1,012
TOTALS FOR MOUNTAIN AREA				
Adult	17	4	212	33
Yearling	5	228	1,696	4,414
Average	22	55	549	1,029
TOTALS FOR ALL ANIMALS				
Adult	76	160	1,393	359
Yearling	70	198	3,546	4,116
Calves	35	195	1,292	442
Average	181	182	2,206	1,828

RESULTS

The average number of parasites found is reported (tables 1 and 3). In the mountain area, the adult animals sampled were

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Contribution from the Department of Animal Industry, North Carolina Agricultural Experiment Station, Raleigh, in cooperation with southern regional research project S-21. Published with the approval of the director of research as paper 755 of the journal series.

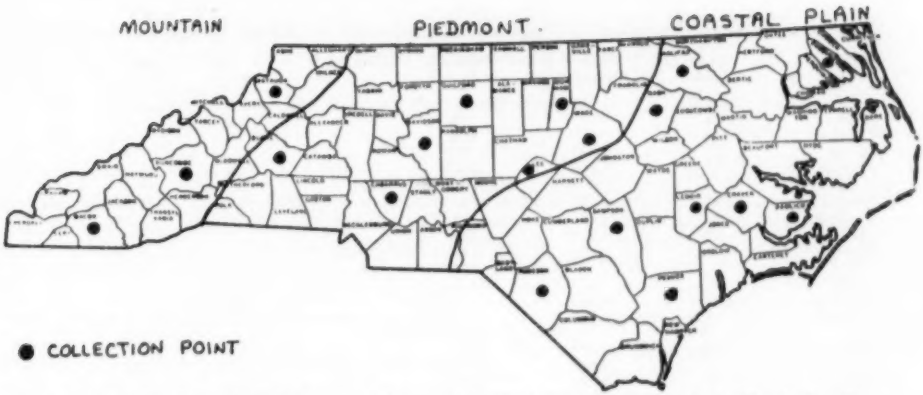

Fig. 1—Collection points for survey of gastrointestinal parasites of cattle in North Carolina in 1955-1956.

TABLE 2—Abomasal Parasites—Percentage of Animals Infected

	Haemonchus contortus				Ostertagia spp.				Trichostrongylus spp.			
Locality	A	Y	C	Total	A	Y	C	Total	A	Y	C	Total
Coastal plain	19	17	70	35	72	86	81	80	59	71	83	71
Piedmont	7	51	21	26	40	86	29	51	37	79	12	43
Mountain	11	80	*	30	55	80	*	67	14	80	*	31

A = adult; Y = yearling; C = calf; * = no collection.

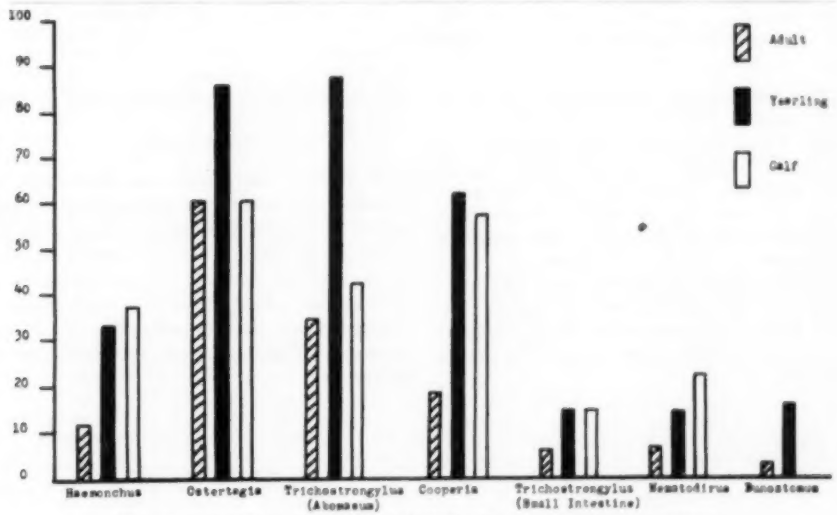

Fig. 2—Percentage of animals infected with parasites by age groups.

TABLE 3—Intestinal Parasites—Average Number Recovered per Animal by Age and Locality

Animal age	No. animals	Cooperia	Trichostrongylus	Nematodirus	Bunostomum	Oesophagostomum
		TOTALS FOR COASTAL PLAIN AREA				
Adult	24	795	36	41	4	247
Yearlings	44	5,397	266	66	36	103
Calves	33	4,307	94	1,056	0	129
Average	91	3,908	162	310	18	149
		TOTALS FOR PIEDMONT AREA				
Adult	35	376	29	0	0	0
Yearling	21	3,616	42	109	23	150
Calves	12	2,423	243	21	0	0
Average	68	1,738	71	37	7	46
		TOTALS FOR MOUNTAIN AREA				
Adult	17	477	0	17	34	2
Yearling	5	4,649	0	0	223	33
Average	22	1,425	0	13	77	9
		TOTALS FOR ALL ANIMALS				
Adult	76	531	25	17	9	78
Yearling	70	4,809	179	74	45	112
Calves	35	2,791	108	171	21	93

mostly beef types in good to excellent condition; therefore, the average number of parasites reported may not be representative of the level of infection in this area.

Abomasum.—Only three genera of parasites were found in the abomasum (table 1). The percentage of animals infected by area and age is shown (table 2).

Small Intestine and Large Intestine.—The average number of parasites found in the intestine (table 3) and the percentage of animals in which they were found are reported (table 4). Six animals were found to harbor 1 Moniezia tapeworm each, while 1 animal had 31 tapeworms and 2 animals had 3 each. One animal from Craven County in the coastal plain harbored 132 parasites of the genus Capillaria.

The only parasite found in the large intestine in large numbers was Oesophagostomum spp. (table 3). Trichuris spp. was found in 7 animals as follows: 3 calves were infected with 23, 2,000 and 6,660, respectively; 3 yearlings with 55, 150, and 124, respectively; and 1 adult with 100.

TABLE 4—Small Intestinal Parasites—Percentage of Animals Infected

Locality	Cooperia				Trichostrongylus spp.				Nematodirus				Bunostomum			
	A	Y	C	Total	A	Y	C	Total	A	Y	C	Total	A	Y	C	Total
Coastal plain	36	56	72	54	10	18	8	12	12	18	23	17	3	8	0	4
Piedmont	13	72	37	41	4	16	25	15	0	20	15	12	0	13	0	4
Mountain	17	100	*	39	0	0	*	0	13	0	*	6	6	60	*	22

A = adult; Y = yearling; C = calf; * = no collection.

SUMMARY

A survey was made to determine the number and species of gastrointestinal parasites of cattle. Specimens were collected, from 19 localities, from 76 adults, 70 yearlings, and 35 calves. The parasites in the abomasum, small intestine, and large intestine were catalogued. The total percentages of animals infected are shown in figure 2.

The Pathogenesis of Trichostrongyloid Parasites
II. Ferrokinetic Studies in Ruminants

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THE DEVELOPMENT of a procedure for determining iron turnover rates in plasma and erythrocytes by Huff *et al.*[1] has resulted in the publication of valuable data concerning the normal rates and changes which result from hematological abnormalities in man.[1-4] In addition, workers at the University of Utah[5-7] have published their work with normal swine and swine suffering from various experimentally induced anemias.

The present study was undertaken to establish certain values relevant to the hemopoietic activity in sheep and cattle as measured in terms of iron turnover rates. In addition to the "normal" animals, an opportunity occurred in which to study the ferrokinetics in a 6-month-old Hereford steer suffering from chronic trichostrongylidosis.

MATERIALS AND METHODS

General Procedure and Calculation of Ferrokinetic Data.—The procedure used in this study was based on the work of Huff *et al.*[1] and Jensen *et al.*[5] An intravenous injection of Fe^{59} was made, after which serial plasma samples were obtained for radioactive analysis. Since Fe^{59} clearance occurs by a first-order process, the results of radioactive assays were plotted on semilogarithmic paper and the $T_{1/2}$ (time at which half of the injected dose had been cleared) determined. In addition to the $T_{1/2}$, extrapolation of the clearance curve to zero time gives the plasma activity at time zero (a_0). From this, the plasma volume (PV) may be calculated by the formula:

$$PV = \frac{TA_I}{a_0}$$

in which TA_I is the total activity injected in counts per minute, a_0 is in counts per minute per milliliter of plasma, and PV is the plasma volume in milliliters.

In the present work, the plasma volume in several of the animals was also determined by the Evans blue dye method. These values were in sufficient agreement to allow the use of either determination for subsequent calculations.

The blood volume (BV) was derived from the formula:

$$BV = \frac{100\ PV}{100\ \% - PCV}$$

no correction was made for body hematocrit.

The hemoglobin (Hb.) levels, packed cell volumes (PCV), and plasma iron levels (PI) were determined by previously described methods.[8,9]

The total erythrocyte iron (RBCI) was found by the equation:

$$RBCI = Hb. \cdot \frac{BV}{100} \cdot 3.4$$

in which Hb. is in grams per 100 ml., 3.4 is the milligrams of iron per gram of Hb., and RBCI is in milligrams.

With the above values the following formulas, taken largely from the work of Huff *et al.*[1] and Jensen *et al.*,[5] were used in calculation of ferrokinetic data.

1) The plasma iron turnover rate (PITR) was found by the equation,

$$PITR = \frac{0.693 \cdot PI \cdot PV \cdot 24}{T_{1/2}}$$

where PI is in milligrams per milliliter, PV is in milliliters, $T_{1/2}$ is in hours, and 24 is the number of hours per day.

2) The percentage of iron incorporated into the erythrocytes was found by determining the blood volume on the day at which radioactivity in the erythrocytes reached a maximum (BVmax), determining the radioactivity per milliliter of blood at this time (Amax), and then applying the formula:

$$Fe^{59}_e = 100 \cdot \frac{BVmax \cdot Amax}{TA_I}$$

in which Fe^{59}_e is the percentage of injected radioactive iron incorporated into the erythrocytes.

3) By applying the formula:

$$RBCIR = PITR \cdot \frac{Fe^{59}_e}{100}$$

the red blood cell iron incorporation rate, (RBCIR) in milligrams per day may be found. If there is no change in blood volume, this will be equal to the red cell iron turnover rate (RBCITR) in milligrams per day.

4) The fraction of erythrocyte iron renewed per day (RBCIRD) may then be found by the equation:

$$RBCIRD = \frac{RBCITR}{RBCI}$$

It is then evident that the reciprocal of this is the red blood cell life span.

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The authors express their appreciation to Mrs. Caroline Pratt for the excellent technical assistance which she supplied during the course of the work presented in this paper.

This work was conducted as part of a contributing project to the western regional project W-35, "Internal Parasites of Ruminants."

As pointed out by Jensen *et al.*,[6] the factor of iron incorporation into erythrocytes as the result of growth must be taken into consideration. In the present study, this was done by determining the daily increase in BV, either by subsequent measurement with Evans blue dye or as a function of body weight, and then calculating the amount of iron required for this increase (IFBVI). This value was then subtracted from the red blood cell iron incorporation rate to give the amount of iron used daily in maintenance of the existing hemoglobin (RBCITR) level. The latter value was used in equation 4 and, consequently, in the determination of the erythrocyte life span.

The Fe^{59} used in this study was obtained from Abbott Laboratories as a sterile solution of ferrous citrate with a specific activity of 1 to 4 $\mu c.$ per microgram. The solution contained an average of 25 to 30 $\mu g.$ of iron per milliliter and had been standardized to contain 100 $\mu c.$ per milliliter.

Preparation of Fe^{59} Labeled Plasma.—Plasma was obtained by drawing blood from normal animals of the same species as that to be studied, into iron-free heparinized syringes. The blood was then centrifuged and the plasma placed in a serum bottle. The latent iron-binding capacity was determined by the method of Rath and Finch[10] and, if it was within the normal range,[8] an appropriate amount was retained in the serum bottle and the excess discarded. The amount to be used was determined by the dosage selected in such a manner that when the radioactive ferrous citrate was added, the latent iron-binding capacity would not be reduced by more than 66 per cent. The dosage used varied from 20 $\mu c.$ to 150 $\mu c.$ depending on the size of the animal. Prior to the intravenous injection of the labeled plasma, a suitable amount (1 to 2 ml.) was removed and diluted to 100 ml. as a standard.

Collection and Preparation of Blood Samples for Analysis.—Blood samples were taken for the determination of Hb., PCV, and PI at the beginning of a trial and again after two to three hours. The determinations were made in duplicate and the mean value for the two samplings was used in the calculations.

During the first 30 minutes after injection, a minimum of three blood samples were collected for radiological assay. In the following five to six hours, an additional three or more samples were taken. Samples were then collected every other day until a maximum incorporation of Fe^{59} into the erythrocytes had occurred.

From each serial blood sample, a 2-ml. sample of whole blood, plasma, and washed cells was placed in a vial 22 mm. in width for radiological assay. Where abnormal sedimentation rates were suspected, a sample was also prepared by washing the cells contained within a given volume of blood three to four times with sterile isotonic saline. The cells were then restored to the original volume with acidified distilled water, after which a 2-ml. sample of the hemolyzed cells was taken. Radiological assay was made by use of a Tracerlab P-20AM scintillation counter connected to a Tracerlab "1000" scaler. This unit gave a counting efficiency of 3.5 per cent with a background count of approximately 300 cpm. (counts per minute).

Animals.—The lambs were raised as previously described.[8] The calves were removed from their dams after receiving colostrum and were then raised on cows' milk and a proprietary calf starter until 1 month of age. At this time, chopped alfalfa hay was added to their ration. Calf 12 was a Jersey male, calf 13 was a Jersey-Guernsey crossbred male, and calf 14 was a Holstein-Friesian male.

TABLE 1—Data from Which Calculations of Ferrokinetics Were Made

Group	Animal No.	Sex	Body weight (kg.)	Growth rate (kg./day)	Plasma volume (ml.)	Blood volume (ml.)	Volume of packed red blood cells (ml./100 ml.)	Hemoglobin (Gm./100 ml.)	Blood volume (ml./kg.)	Plasma iron ($\mu g.$/100 ml.)
Lambs, 3 months old	251	Wether	10.4	0.15	479	694	31	9.8	75	185
	252	Wether	9.8	0.18	494	737	33	10.2	67	166
	218	Ewe	11.8	0.03	479	748	36	11.8	63	222
Mean	.....	.....	10.7	0.12	484	726	33	10.6	68	191
Lambs, 1 year old	4389	Wether	41.0	0.11	1,718	2,390	28	9.9	58	150
	4371	Wether	36.0	0.11	1,275	1,960	35	11.4	55	170
	4213	Wether	36.0	0.08	1,209	1,860	35	11.6	52	152
	25	Wether	31.0	0.09	1,166	1,770	34	10.9	57	120
	113	Wether	38.6	0.05	1,337	2,025	34	10.3	52	111
Mean	.....	.....	36.5	0.09	1,341	2,001	33	10.8	55	141
Calves, 3 months old	12	Male	56.8	0.52	2,076	3,243	36	10.5	57	275
	13	Male	64.1	0.49	2,497	3,619	31	9.8	57	225
	14	Male	79.5	0.49	3,175	4,885	35	10.5	61	192
Mean	.....	.....	66.8	0.5	2,853	3,916	34	10.3	58	231
Hereford, 6 months old, "trichostrongylidosis"	.....	Steer	96.4	0	5,673	7,464	24	6.8	77	170

The 6-month-old Hereford steer suffering from chronic trichostrongylidosis, principally *Ostertagia ostertagi* infection, was obtained from a herd which had recently been severely affected with this disease.

RESULTS

The data from which the ferrokinetic values were calculated are presented in table 1, while the ferrokinetic data are presented in table 2. The ranges for the plasma Fe^{59} clearance and erythrocyte Fe^{59} uptake rates are illustrated in figures 2 and 3, respectively.

With the exception of 1 animal, lamb 25, all animals showed a two-component clearance curve for Fe^{59} in plasma (fig. 1). It has been demonstrated by Jensen *et al.*[5] in swine that the first component of the clearance curve is closely related to liver uptake of isotope and, like Huff *et al.*,[1] they have taken the second component to be representative of bone marrow uptake. The same assumption was made in the present study, and the $T_{1/2}$ was determined from the second component.

Lambs Three Months of Age.— The $T_{1/2}$ values for lambs 3 months of age were 1.25, 1.33, and 2.08 hours, with a mean of 1.55 hours. This indicates that an iron turnover equivalent to the total plasma iron would occur on an average of 10.7 times every 24 hours. In 2 of the 3 lambs, the maximum uptake of Fe^{59} into erythrocytes had occurred by the sixth day, whereas in the third animal, 12 days were required.

Of the total average plasma iron turnover, 10.2 mg. per day, a mean of 85 per cent, was incorporated into red blood cells. Further calculation revealed that of this amount, 5.6 mg. was used to maintain the amount of hemoglobin already present, while 3.1 mg. was used in the expansion of blood volume. Consequently, it was found that 0.0212 of the total red blood cell iron was being renewed each day and that the erythrocyte life span averaged 46 days.

Lambs One Year of Age.—The $T_{1/2}$ values for the 5 lambs 1 year of age ranged from 1.42 to 1.83 hours, with a mean of 1.57 hours, indicating a complete plasma iron turnover 10.6 times every 24 hours. The maximum uptake of Fe^{59} by erythrocytes occurred between the twelfth and fifteenth day after injection. The mean uptake of isotope by red blood cells was 78 per cent. The total average plasma iron turnover was 20.4 mg. per day, of which 15.9 mg. was incorporated into erythrocytes. Of this, 1.8 mg. went into the formation of an increased blood volume, while 14.1 mg. was used to replace existing hemoglobin. This represents a turnover of 0.019 times the total red blood cell iron and indicates an erythrocyte life span of 52 days.

Calves Three Months of Age.—The $T_{1/2}$ values for the 3 calves 3 months of age ranged from 1.75 to 1.97 hours, with a mean of 1.82 hours. This indicates that the total plasma iron was removed and

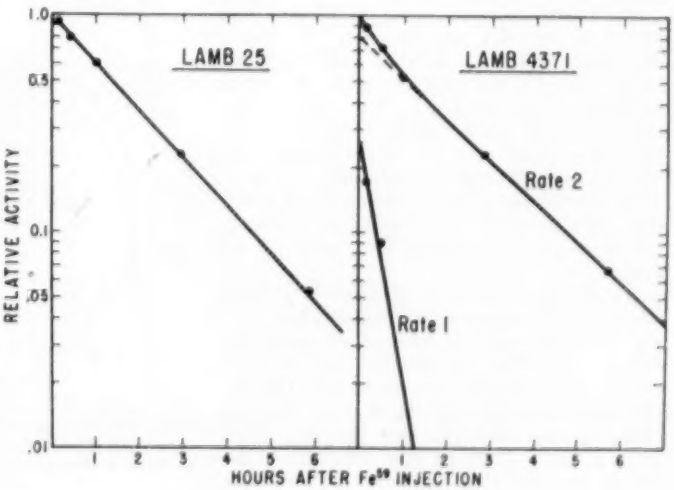

Fig. 1—Representative plasma clearance curves of one component, lamb 25, and two components, lamb 4371. Relative activity is the fraction of injected Fe^{59} remaining in plasma at any time. Only 1 of 12 animals was found to have a one-component clearance rate. In the case of those having two components, the second component was used for determination of the $T_{1/2}$.

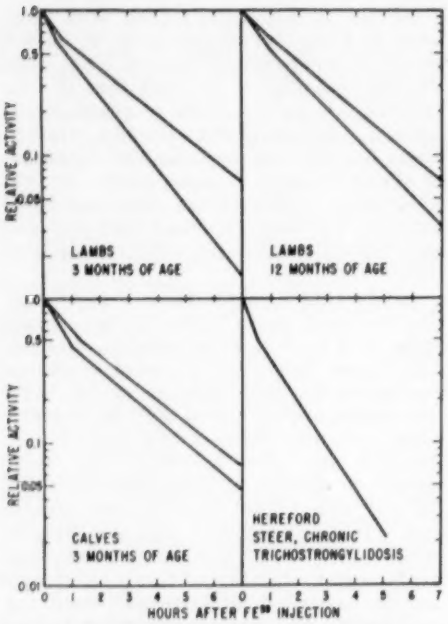

Fig. 2—The Fe^{59} plasma clearance rates. The areas encompassed include the range of data found for the various groups. Relative activity is expressed as the fraction of injected Fe^{59} remaining in plasma at any time.

replaced 9.1 times every 24 hours. The maximum uptake of isotope by erythrocytes occurred between nine and 12 days after injection and represented a mean of 68 per cent of the injected dose.

The total plasma iron turnover was found to be 54.1 mg. per day of which 36.8 mg. was incorporated into erythrocytes. As in the young lambs, a large portion of this, 10.6 mg., was used in the expansion of blood volume. As a result, 26.2 mg. was used in maintaining the amount of hemoglobin already present. This represents a daily turnover of 0.018 times the total red blood cell iron, indicating a mean erythrocyte life span of 55 days.

Hereford Steer Six Months Old with Chronic Trichostrongylidosis.—This animal was showing an anemia of moderate degree (6.8 Gm. of Hb./100 ml., PCV of 24%). The plasma iron level was 170 μg. per 100 ml. and the total iron-binding capacity was 260 μg. per 100 ml. This gives a figure of 90 μg. per 100 ml. for the latent iron-binding capacity and 65 per cent for the saturation percentage. Unpublished data from this laboratory indicate that calves may have normal plasma iron values ranging from 150 to 300 μg. per 100 ml., with total iron-binding capacity of 300 to 400 μg. per 100 ml. It therefore appears that the total iron-binding capacity, and perhaps the latent iron-binding capacity, was somewhat below normal, while the plasma iron level was normal.

The $T_{1/2}$ for plasma clearance was 1.04 hours, representing a complete turnover of plasma iron every one and one-half hours. The total plasma iron turnover was 152 mg. per day, of which 100 per cent had entered the erythrocytes by the ninth day. Since the animal did not change its blood volume, all of this iron was utilized in maintaining the amount of hemoglobin present at the start of the trial. A red blood cell iron turnover rate of this magnitude would result in the turnover of 0.089 times the red blood cell iron daily and a mean erythrocyte survival of only 11.0 days.

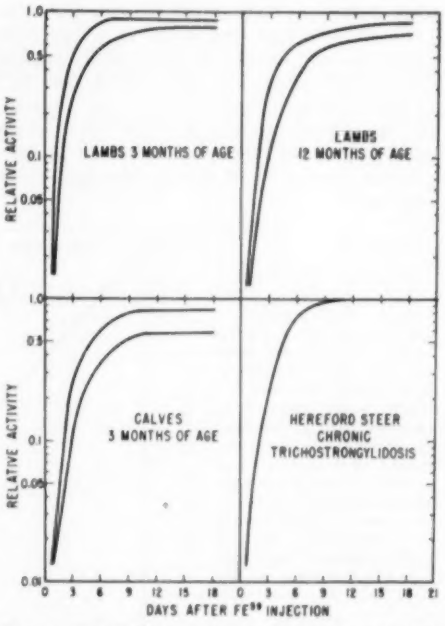

Fig. 3—The Fe^{59} incorporation rate into red blood cells. The area encompassed includes the range of values for the various groups. The relative activity is the fraction of injected Fe^{59} in the circulating erythrocytes.

Discussion

Studies on the pathogenesis of the anemias produced by members of the Trichostrongyloidea have been largely confined to routine hematological examinations of peripheral blood. This type of study has established that the anemia resulting from *Haemonchus contortus* infection is principally due to gastric hemorrhage. Little information, however, has been obtained with regard to the pathogenesis of the anemias which result from infection by other members of this group. It would appear that if the mechanism of these anemias is to be revealed, a new approach must be employed. An approach which offers much promise is the study of the metabolism and metabolic rates involved in hemopoiesis. Fortunately, a tool has been provided in the form of isotopic radioactive iron.

Due to the paucity of information concerning these rates in ruminants, it was necessary to obtain information regarding normal values. As would be expected, the red blood cell iron incorporation rates in young lambs and calves were much greater than in mature lambs (table 2). This difference between older lambs and calves was due entirely to the expansion of blood volume in rapidly growing animals. The difference between the younger and older lambs was, in part, also due to the fact that the younger lambs had a greater blood volume per kilogram of body weight (table 1). This latter factor is exemplified in the amount of iron used for maintenance of existing hemoglobin (column 7, table 2).

In view of the wide ranges, there is probably no significant difference between the values found for the erythrocyte life span in any of the three groups of "normal" animals and, consequently, the life span in sheep and in cattle is comparable. No reports concerning the life span of erythrocytes in either sheep or cattle could be found with which to compare the present results.* Huff *et al.*[1,2] found good agreement in the data obtained from normal persons and others by the ferrokinetic method and other reported procedures. In addition, Jensen *et al.*[5] with swine, although encountering a wide range, found a mean of 63 days with the ferrokinetic method which compared well with that of 62 days reported by Bush *et al.*,[11] as determined with glycine-2-C^{14}.

It appears that when ferrokinetic data from lambs and calves are based on a unit of body weight, the normal values are comparable. In order to make data from all

* Hansard and Kincaid have recently published an abstract of their work with chromium 51 in the determination of red blood cell life span of farm animals (*J. Anim. Sci., 15,* [1956]: 1300). They report an average of 71 ± 10 days for swine, whereas burros, cattle, and sheep averaged 70 to 80 per cent of this.

TABLE 2—Ferrokinetic Data

Group	Animal No.	Sex	PITR* (mg./kg./day)	Fe^{59} † (%)	RBCIIR‡ (mg./kg./day)	IFBVI§ (mg./kg./day)	RBCITR‖ (mg./kg./day)	Erythrocyte life span (days)
Lambs, 3 months old	251	Wether	1.07	86	0.92	0.32	0.6	37
	252	Wether	1.11	90	1.0	0.48	0.52	50
	218	Female	0.72	80	0.58	0.06	0.52	50
Mean	.....	.....	0.97	85	0.83	0.29	0.55	46
Lambs, 1 year old	4389	Wether	0.57	76	0.43	0.05	0.38	49
	4371	Wether	0.65	74	0.48	0.07	0.41	53
	4213	Wether	0.63	87	0.55	0.05	0.5	40
	25	Wether	0.53	81	0.43	0.06	0.37	56
	113	Wether	0.42	83	0.31	0.01	0.3	63
Mean	.....	.....	0.56	78	0.44	0.05	0.39	52
Calves, 3 months old	12	Male	0.95	65	0.62	0.19	0.43	48
	13	Male	0.83	57	0.47	0.1	0.37	53
	14	Male	0.65	82	0.53	0.18	0.35	63
Mean	.....	.....	0.81	68	0.54	0.16	0.38	54
Hereford, 6 months old, "chronic trichostrongylidosis"	.....	Steer	1.58	100	1.58	0	1.58	11

* PITR = plasma iron turnover rate. † Fe^{59} = percentage of injected Fe^{59} incorporated into red blood cells. ‡ RBCIIR = red blood cell iron incorporation. § IFBVI = iron required for blood volume expansion. ‖ RBCITR = iron used for maintenance of existing hemoglobin, or red blood cell iron turnover rate.

individual animals comparable, it would seem that the great variance in blood volume per kilogram of body weight which occurs in domestic animals should be considered. This might be done by expressing the red blood cell iron turnover rate in terms of a standard blood volume per kilogram of body weight. For example, if a standard value of 60 ml. per kilogram is selected, then the mean values (column 7, table 2) become 0.48, 0.43, 0.39, and 1.23, respectively. Values determined in this way might better express the functional activity of the bone marrow for comparison between animals, particularly in those suffering from hematological disorders accompanied by increased or decreased blood volume per kilogram of body weight.

As an example of information which may be gained regarding the anemias of trichostrongylidosis by employment of ferrokinetic methods, the following discussion of data from the Hereford steer is presented.

Anemia, exclusive of that due to hemorrhage, may be produced in three major ways as the result of physiological alterations. These are: (1) a reduction in the amount of functional hemoglobin being released by the bone marrow into the blood stream, (2) a reduction in the erythrocyte life span of sufficient magnitude that the bone marrow can not compensate, and (3) a combination of the two. It has been demonstrated [6] that normal swine can increase their bone marrow capacity four to five times. Therefore, in order to produce anemia in these animals if no inhibition of bone marrow function were present, it would be necessary to shorten the life span of the erythrocytes by four to five times. If the ability of the bone marrow to expand its function were reduced, then the shortening of that life span required for the production of anemia would be proportionately less.

The erythrocyte life span in the Hereford steer had been shortened to approximately one fifth of its normal value (table 2). In addition, based on the erythrocyte iron turnover rate, there was a marked increase in the rate of hemoglobin formation. This increase was approximately fourfold. If, however, the amount of increase is based on a standard blood volume per kilogram of body weight, as suggested above, the increase would approximate a factor of 3. While no data are available on the ability of cattle to increase their bone marrow activity, it is reasonable to assume that it might approximate that of swine [6] and man.[4] If this is the case, there was some reduction in the ability of the bone marrow to increase its red cell production, and the anemia was due to the decreased erythrocyte life span and the inability of the bone marrow to compensate for this.†

The fundamental cause of the reduction in erythrocyte life span is not known; however, there are certain conditions which deserve consideration. The erythrocyte survival time in various nutritional deficiencies has been summarized as follows [6]: copper and pteroylglutamic acid deficiency in swine produces a decreased life span, as does vitamin B_{12} and ascorbic acid deficiency in man. In pyridoxine deficiency of swine, the life span of erythrocytes is normal. Bush *et al.*,[7] in a later paper, reported that the mean survival time of erythrocytes from iron-deficient swine when transfused into other iron-deficient swine was normal.

With the exception of ascorbic acid deficiency, it appears possible to eliminate all of the above-mentioned deficiencies as being the sole cause of the anemia in the Hereford steer. This, of course, must rest on the assumption that cattle would show the same hematological and ferrokinetic changes as swine and man when subjected to the same deficiencies. Iron deficiency is eliminated by the normal plasma iron level, and the markedly increased iron turnover rate. In copper deficiency, as in iron deficiency, the plasma iron levels are markedly lowered and the ability of the bone marrow to compensate for the anemia is greatly reduced. As a result, the red blood cell iron incorporation rate is increased only from 1.1 to 1.3 times.[7] In pteroylglutamic acid and vitamin B_{12} deficiencies, the plasma iron levels are increased, the rate of iron incorporation into erythrocytes is decreased, and the amount of Fe^{59} which eventually enters the red blood cells is reduced.

† Since preparation of this manuscript, the writers have had an opportunity to study 2 lambs suffering from chronic trichostrongylidosis. In both of these animals, the plasma iron components were within normal ranges. The hemoglobin levels were 8.6 and 9.4 Gm./100 ml. The red blood cell iron turnover rates were 0.92 and 1.2 mg./kg./day, while the calculated erythrocyte survival time was 17 and 22 days. These data lend support to that from the Hereford steer concerning the mechanism of anemia in this condition.

In ascorbic acid deficiency, the plasma iron level is normal,[5] whereas the erythrocyte survival time is decreased, a situation comparable to that in the present case. However, no reports of ferrokinetic studies of this condition could be found with which to compare the present results.

An additional condition which in many ways resembles that seen in the present case of chronic trichostrongylidosis is termed infection anemia. This condition, while being accompanied by a decrease in total plasma iron-binding capacity, is also accompanied by a marked drop in plasma iron. It has also been demonstrated that the plasma iron turnover rate in man is slightly increased,[4] reaching 0.64 mg. per kilogram per day as compared to a normal value of 0.48 mg. per kilogram per day. The red blood cell iron renewal rate was 1.9 per cent per day in the same patients as compared to 1.2 per cent in the normal individuals. This would represent a shortening of the red blood cell survival time to 60 per cent of normal and an increase in hemoglobin formation of only 1.3 times. Therefore, it would appear that the major cause of infection anemia is the inability of the bone marrow to compensate for the moderately shortened life span of the red blood cells.

Experimental data which closely correlate with those seen in the present animal have been reported in swine[6] in which hemolytic anemia was produced by phenylhydrazine injection. In that group (3 animals), the mean hemoglobin level was reduced to 6.8 Gm. per 100 ml. The plasma iron was increased in 1 animal and decreased in 2, although the mean value was normal. The plasma iron turnover was greatly increased as was the rate of Fe^{59} incorporation into the red blood cells. The fraction of injected Fe^{59} incorporated into the erythrocytes was similar to that in control animals. The amount of iron incorporated into red blood cells was 4.2 times that of the control animals and the mean life span of erythrocytes was reduced from 63 to five days.

It is very unlikely that any hemolytic products produced by the parasite were responsible for the reduced life span of erythrocytes in the Hereford steer. Therefore, it is probable that the factor was either intrinsic, in which case the cells were produced with a condition which subjected them to early hemolysis, or there was some environmental influence on the integrity of the cell, or both. That it could be an environmental influence and result from some undefined deficiency is exemplified by the findings of Bush *et al.*[7] with copper deficiency in swine. These workers found that when the red blood cells from copper-deficient animals were transfused into copper-deficient recipients the survival time was significantly shorter than that of cells transfused from normal to normal swine. It was also found that when red cells from the copper-deficient animals were transfused into normal recipients the mean survival time returned toward normal. Furthermore, Hamilton *et al.*[12] working with pernicious anemia, and Merskey[13] with ascorbic acid deficiency in man, found that when normal blood was transfused into their untreated patients there was a shortening of the expected survival time. The former workers found that the administration of vitamin B_{12} daily for nine days prior to transfusion prevented the hemolytic action. A similar observation was made by Merskey in certain of his ascorbic acid-deficient patients.

Another condition which may prove to deserve consideration is symptomatic hemolytic anemia. This process is associated with some primary disease and results from the formation of autoimmune antibodies[14] which are active against the red blood cells of the host.

In view of the foregoing discussion, it is concluded that the anemia in this particular animal, and perhaps in many such animals suffering from chronic trichostrongylidosis, was principally due to the fivefold shortening of erythrocyte survival time and to some inhibition in the ability of bone marrow to increase its hemoglobin production. It is postulated that this condition may well have been due to some specific nutrient deficiency, or to a multiple deficiency resulting from the activity of the parasites. The possibility of autoimmune anti-red blood cell antibody formation, as well as other factors, can not be ruled out, however, and it will be necessary to examine many more such animals using the present and additional techniques before the underlying cause is definitely established.

SUMMARY

By use of Fe^{59}, the plasma and red blood cell iron turnover rates were determined in 3 lambs, 3 months old; 5 lambs, one year old; 3 calves, 3 months old; and 1 steer, 6 months old, which was suffering from chronic trichostrongylidosis.

The mean plasma iron turnover rate in the lambs 3 months of age was 0.97 mg. per kilogram per day of which 85.0 per cent was incorporated into erythrocytes. The average erythrocyte life span was calculated to be 46 days.

The mean plasma iron turnover rate in the 1-year-old lambs was 0.56 mg. per kilogram per day of which 78.0 per cent was incorporated into erythrocytes. The mean erythrocyte survival time was calculated to be 52 days.

The mean plasma iron turnover rate in the calves 3 months old was 0.81 mg. per kilogram per day of which 68.0 per cent was incorporated into the erythrocytes. The mean erythrocyte life span in these animals was 54 days.

In the steer suffering from chronic trichostrongylidosis, the plasma iron turnover rate was 1.58 mg. per kilogram per day which represented an increase of three to four times that of the control animals. Of this, 100 per cent was incorporated into the red blood cells at a somewhat accelerated rate. The calculated life span of the red blood cells was found to be only 11 days.

As the result of the hematological and ferrokinetic data, it was concluded that the anemia in the steer was due principally to the fivefold shortening of red blood cell survival time and to inhibition in the ability of the bone morrow to compensate for this. It is postulated that the activity of the parasites may have produced either a plasma environment unsuitable to the integrity of erythrocytes, or an intrinsic flaw in the red blood cell at the time of its formation which resulted in early hemolysis, or both. Fundamental alterations resulting in the decreased red blood cell survival time may possibly have resulted from a nutrient deficiency or some condition such as symptomatic hemolytic anemia.

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Enhancement of Listeriosis in Sheep with Material from Bovine Mucosal Disease

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THE DEMONSTRATION of a relatively mild pathogenic agent capable of enhancing the effect of *Listeria monocytogenes* in experimental sheep [1] has led to a search for similar agents. Because sheep have been incriminated as asymptomatic carriers of the agent of malignant catarrhal fever,[2-5] we had hoped to test material from cattle with malignant catarrhal fever. Listeria have been isolated from cases of malignant catarrhal fever, in one instance from the nasal passage [6] and in two instances from the brain.[7] One author considered Listeria an etiological factor [6] but the other [7] believed Listeria incidental and that listeriosis and malignant catarrhal fever were distinct entities which often were confused. Malignant catarrhal fever occurs relatively infrequently and we have been unable to obtain satisfactory material for test. Bovine mucosal disease, which has many features similar to bovine malignant catarrh, has occurred in our area, so we have directed our efforts accordingly.

Material from cattle with mucosal disease, as well as from a second outbreak of listeriosis in sheep, has shown a similar enhancing effect. These observations constitute the basis for this report.

METHODS

Sheep were used to demonstrate agents capable of assisting the Listeria organism in establishing itself in the brain stem. No other experimental animals were found satisfactory for this purpose.[1]

The experimental sheep were 6 to 17 months old and most of them were raised on University premises. Although listeriosis has occurred occasionally in the flock, no experimental animals were obtained while the disease was evident. *Listeria monocytogenes* (strain 1501-2) was used in conjunction with ground tissues whose activity in enhancing listeriosis was being examined. In the earlier experiments, the lambs were given a mixture of Listeria culture and ground tissue intravenously and tissue intranasally. The methods of exposure will be referred to as "intravenous" and "intranasal" in the experiments.

The techniques of exposure, leukocyte counts, and bacteriological methods are given in detail in another report and need not be repeated here.[1]

MATERIALS AND RESULTS

Brief descriptions of the source of each material tested, the trials made, and results obtained are presented separately for each material.

Materials L207-1 and L207-3.—In a herd of 180 Hereford yearling calves, during a period of five months, 53 calves were affected and 43 died. The signs and lesions were characteristic of mucosal disease. Material L207-1 consisted of lymph nodes from the region of the head and neck from a calf with a typical case which died during the third month. Material L207-3 consisted of lymph nodes from the pharyngeal region and mesenteric lymph nodes of a calf which died after an illness of seven to ten days near the end of the outbreak. The history of this herd is reported in greater detail elsewhere (herd A).[8]

Two sheep were given L207-1 lymph node both intranasally and intravenously with Listeria (table 1); 1 died in four and the other in six days. Listeria organisms were isolated from both the central nervous system and the visceral organs.

The pharyngeal and parotid lymph nodes (L207-3) were given to 2 test sheep (113 and 149, table 1) and mesenteric lymph nodes (L207-3) were given to 2 other sheep (128 and 154, table 1). The suspected materials were given intranasally and intravenously with Listeria. One of the 4 sheep died with listeriosis 36 days after exposure. The body temperature became elevated within a few hours after exposure and continued for two days in 1 animal and for seven days in the other 3. Since only one of the 4 test animals

From the Department of Animal Pathology and Hygiene, University of Nebraska, Lincoln. Published with the approval of the director as paper 790, journal series, Nebraska Agricultural Experiment Station.

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This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service.

developed listeriosis, material L207-3 must be considered without enhancing effect.

Thus an apparent enhancing effect of material from this mucosal disease source was obtained only with L207-1.

Material L65-2.—A specimen of saliva came from the second of 2 cases of typical mucosal disease which developed, about three months apart, in a group of 40 Aberdeen Angus steers in a feedlot. Both died after an illness of about ten days.

Two sheep were given the saliva, which had been stored at —60 C., both intranasally and intravenously together with Listeria (table 1). One of them showed circling, inappetence, and a preference to lie down 19 days after exposure; when killed, 24 days after exposure, Listeria could not be isolated from the central nervous system or the viscera. There was no histological evidence of Listeria infection in the brain stem, cerebrum, cerebellum, or spinal cord. These sheep had a bacteremia lasting for two and six days after exposure and a temperature elevation lasting for two and 12 days postexposure.

Although the circling was suggestive evidence of Listeria infection of the central nervous system, this could not be confirmed by attempts at culture isolation or histological study. The material was considered as having no enhancing effect on listeriosis.

*Material ISC-1.**—This consisted of cervical lymph nodes, preserved in 50 per cent glycerine and kept at 5 C., obtained from a steer which died with mucosal disease. It was 1 of 35 Hereford steers in an Iowa feedlot where 3 others had died with mucosal disease. The steers had been raised on a ranch in Nebraska adjacent to the ranch where L207 material originated. However, these steers had been in Iowa about a year before the disease developed.

When ISC-1 material was received, it was injected both intranasally and intravenously with Listeria into a test sheep (118, table 1) which died four days later. Listeria were isolated from both the central nervous system and the viscera. Two months later, the ISC-1 material was injected similarly into another test sheep (111, table 1) which remained well.

Lymph node material from the sheep which died was injected into 2 aged ewes (646 and 647, table 1), 1 of which developed typical listeriosis and died 20 days after inoculation. Listeria were isolated from the cerebrum, cerebellum, brain stem, and lumbar cord, whereas the brachial cord and visceral organs were negative. The same lymph node material was injected into the prescapular lymph node and sprayed in the nostrils of a 4-month-old calf. Listeria were present in the lymph node and no cultured organisms were added to the inoculum. The calf's prescapular lymph node swelled to about three times its normal size for about two weeks. A marked nasal discharge developed in three days, disappeared after

* Materials and history were received through the courtesy of Drs. F. K. Ramsey and W. M. Monlux, Department of Veterinary Pathology, Iowa State College, Ames.

TABLE 1—Summary of Attempts to Demonstrate Listeriosis-Enhancing Effect of Various Materials from Cattle with Mucosal Disease

Material	Sheep No.	Exposure route	Bacteremia	First signs	Death	Listeria isolated
L207-1	162	i.n. and i.v.	1 through 6*	1*	6*	+
	174	i.n. and i.v.	1 through 4	1	4	+
ISC-1	118	i.n. and i.v.	1 through 3	2	4	+
	111	i.n. and i.v.	1 through 5	—	8	—
	646	i.n. and i.v.	1 through 2	19	20	+
	647	i.n. and i.v.	1 through 2	....	8	—
M833	106	i.n.	2nd	23	36	+
	130	i.n.	7th	25	27	+
L207-3	149	i.n. and i.v.	1 through 5	....	8	—
	113	i.n. and i.v.	1 through 4	....	8	—
	128	i.n. and i.v.	1 through 2	....	8	—
	154	i.n. and i.v.	1 through 5	36	36	+
L65-2	92	i.n. and i.v.	1 thro. gh 5	20	24	—
	138	i.n. and i.v.	1 through 3	....	8	—
ISC-2	650	i.n.	....	....	8	—
	651	i.n.	....	....	8	—
	654	i.n. and i.v.	1	....	8	—
	655	i.n. and i.v.	1	....	8	—
M150	672	i.n.	....	....	8	—
	675	i.n.	....	....	8	—

i.n. = intranasal; i.v. = intravenous; 8 = survived.
* Expressed in days from time of exposure.

four days, and recurred from the tenth to fourteenth days. On the twenty-ninth day, the calf was depressed and, on the thirty-first day after exposure, had definite signs of a central nervous system disturbance. It preferred to lie but could not be made to lie on the right side. When forced to stand, it circled continuously, always turning to the left (fig. 1). The right ear was paralyzed and reflexes were absent in the right eyelid. There was a thick, grayish mucous discharge from the nose and excessive salivation (fig. 2). Since its condition was critical, it was killed for necropsy. Various portions of the brain and roots of the cranial nerves were cultured for Listeria, as well as various lymph nodes and visceral organs. Only the root of the left trigeminal nerve yielded Listeria. There were definite histological lesions of listeriosis in the brain stem.

Lymph node material from the calf was injected intranasally and intravenously, with Listeria, into 3 sheep. Since all 3 lived, an enhancing agent could not be demonstrated in the lymph node of the calf. Blood from the calf, taken ten days after it had been exposed and at a time when it was showing a moderate increase in temperature, was given in a like way with Listeria to a test sheep. The test sheep died with listeriosis in six days. The calf was previously infected with *Haemonchus contortus* as part of an unrelated experiment. Several of these parasites were collected at time of necropsy and stored in 50 per cent glycerine. The parasites were ground and the suspension was injected intranasally and intravenously with Listeria into a test lamb. The lamb died nine days later with listeriosis.

Thus, the material (ISC-1) from the case of mucosal disease appeared to enhance listeriosis in one test (the calf) but, after two months' storage, it was inactive. An enhancing agent was probably in the blood of the calf ten days after exposure. It appeared to be in nematodes of the calf's small intestine but not in a lymph node at time of death.

Material ISC-2.—This material was the prescapular and prefemoral lymph nodes from a 6-month-old Shorthorn which died after eight days of illness. The heifer was one of 27 kept on pasture, 5 of which died from mucosal disease.

The glycerinated lymph node material and Listeria were injected intranasally into 2 sheep and both intravenously and intranasally into 2 other sheep (table 1). While a bacteremia was established in the 2 which were given the material intravenously and the body temperatures of all 4 sheep became elevated, the material

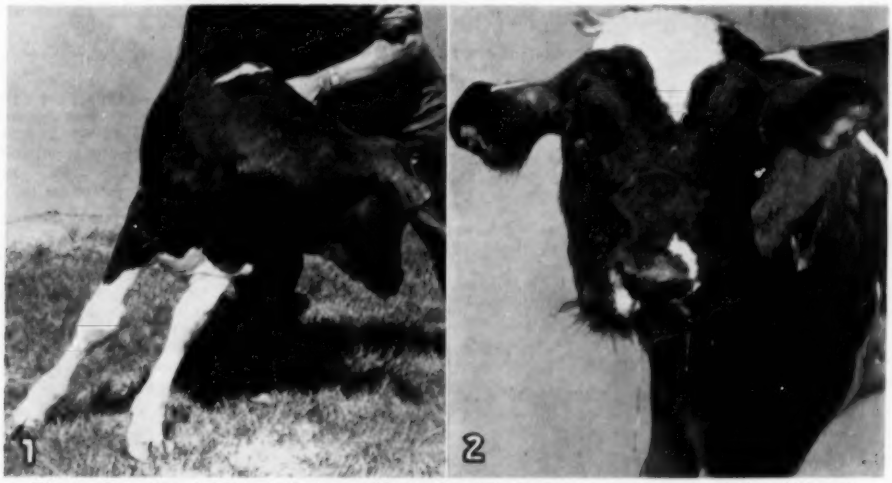

Fig. 1—Experimental listeriosis in a calf showing imbalance and tendency to circle. Although its strength was adequate, the calf could not stand without support.
Fig. 2—Nasal discharge and salivation of the same calf as shown in figure 1. Facial paralysis of the right side was also marked.

was considered as not having an enhancing effect since none died.

Material M150.—This material consisted of glycerinated lymph nodes from a steer with an undiagnosed illness; 4 of 25 steer calves which were kept in this feedlot developed symptoms of lameness, depression, salivation, nasal discharge, and anorexia followed by a profuse diarrhea and rapid emaciation. Two of the sick calves were sent to slaughter early in the disease but were condemned as unfit for food. Another calf, brought to the laboratory for examination, had a marked leukopenia (about 600 leukocytes/cmm. of blood consisting almost entirely of lymphocytes with very few neutrophils). The gross and microscopic pathological changes were only suggestive of mucosal disease; a specific diagnosis could not be made.

A ground suspension of glycerinated lymph node material from the latter calf was injected intranasally with Listeria into 2 susceptible test sheep (table 1). No bacteremia was established and there were only mild changes of body temperature and circulating leukocytes. The sheep remained alive and the material was considered as without enhancing effect.

Material M833.—This material consisted of lymph nodes and other tissues from calves which died from mucosal disease and also blood taken from calves of the same herd which had elevated temperatures and some signs of mucosal disease. This epizootic was in a herd of 223 Hereford calves and lasted for five months, during which time 31 died with the disease. Details of the history are given more fully in another report (herd B).[5]

This ground glycerinated lymph node material and blood was mixed with a broth culture of Listeria and given intranasally to 2 sheep (table 1). Both sheep developed typical listeriosis and died 27 and 36 days after the exposure. Listeria were isolated from both (from the central nervous system of 1, the viscera and central nervous system of the other). A mild bacteremia lasting for only one day occurred in both sheep (the second postinoculation day in 1, the seventh day in the other). A moderate initial rise of body temperature in both animals lasted for two days in 1 and seven days in the other sheep. Temperatures again were elevated on the twenty-fifth and twenty-seventh days postexposure. A slight lymphopenia developed in both animals and a moderate neutrophilia in 1 (fig. 3). This material had a clearly demonstrable, enhancing effect.

Material LEA-2.—This material originated in a flock of 700 lambs, 1 of which died with listeriosis. Clinical listeriosis was not observed among the remainder, although the herdsman was well acquainted with the disease from previous experience.

Previous work [1] had indicated that lambs with elevated temperatures, but no signs of the disease, might have an enhancing

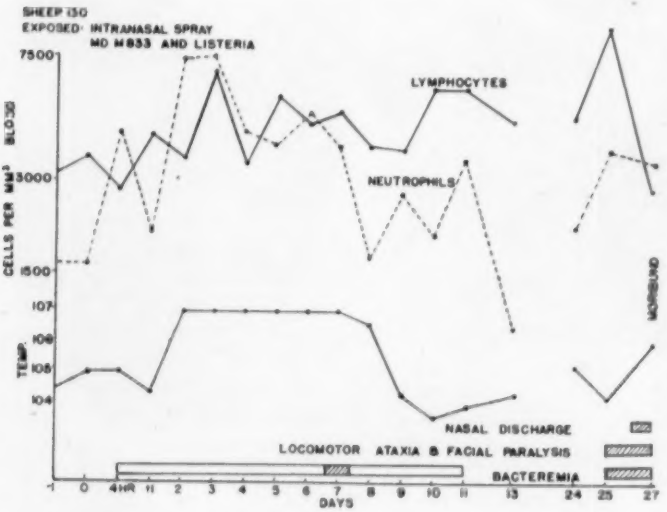

Fig. 3—Graphic representation of experimental listeriosis in a sheep which received Listeria and mucosal disease material intranasally.

Note the early increase of neutrophils and temperature lasting for six and seven days, respectively (a somewhat longer initial reaction than usual), and essentially normal value from the ninth to twenty-fifth days when symptoms developed. Blood was cultured daily for Listeria during the first 11 days, and again on the twenty-fifth and twenty-seventh days (bottom bar).

Cross-hatched areas indicate positive findings.

agent in their blood. Temperatures were taken daily on 160 of the lambs for three days. Blood taken from 4 lambs, with temperatures greater than 106 F., was frozen and kept stored at —65 C. until used.

Portions of the blood from each of the 4 lambs were pooled and injected intranasally with Listeria into 2 test sheep. One developed paralysis of the jaw on the sixteenth day, complete left facial paralysis two days later, and died on the twenty-first day. Listeria were isolated from the cerebrum, medulla, and root of the trigeminal nerve. A dyspnea developed in the other sheep on the eighteenth day and marked depression on the twenty-third day; it died on the twenty-fourth day. Listeria were isolated from the cerebellum, medulla, and right trigeminal nerve root.

Blood was again taken from the same 4 lambs (their temperatures were then normal), one month after the original sampling. The blood was frozen and handled in the same manner as previously. Two test sheep were intranasally exposed with a mixture of the composite blood and Listeria. Both remained alive and no symptoms of listeriosis developed.

Work with this material confirms the previous observation of an enhancing agent existing in sheep where listeriosis has occurred. It further indicates that the enhancing agent may be relatively transient in the blood stream.

Discussion

Some of the tests for enhancing of listeriosis were done by giving sheep the suspected material both intranasally and intravenously with *L. monocytogenes*. It was presumed that an enhancing agent was present when the sheep died with listeriosis in four to six days. This test is less critical than if the suspected material and Listeria are given intranasally, since some sheep exposed intravenously to Listeria alone may develop listeriosis.[1] However, a negative result after combined intravenous and intranasal exposure is definite evidence that an enhancing agent is not present. Such a result was obtained with materials from three affected herds (207-3, ISC-2, and L65-2, table 1).

Material from animals in three herds with mucosal disease (L207-1, ISC-1, and M833) gave evidence of enhancing listeriosis in the experimental sheep (table 1). The more critical test of intranasal exposure was not done with material from two of the sources (L207-1 and ISC-1). Material from two other mucosal disease sources (ISC-2 and L65-2) failed to dem-

TABLE 2—Materials Screened for Enhancing Effect with Listeria Monocytogenes by Exposure of Sheep

Source	Material	Trials	Sheep	Listeriosis	
				Positive	Negative
	MATERIALS WITH ENHANCING EFFECT				
Listeriosis (sheep)	Blood	5	18	15	3
Listeriosis (sheep)	Blood	1	2	2	0
Mucosal disease	Lymph node	1	2	2	0
Mucosal disease	Lymph node	3	5	4	1
Mucosal disease	Blood and lymph node	1	2	2	0
Totals		11	29	25	4
	MATERIALS WITHOUT ENHANCING EFFECT				
Listeriosis (sheep)	Blood	1	2	—	2
Listeriosis (sheep)	Blood	2	3	—	3
Postlisteriosis	Blood	1	2	—	2
Listeriosis (cattle)	Blood	1	2	—	2
Shipping fever	Blood	1	1	—	2
Mucosal disease	Lymph node	1	4	1	3
Mucosal disease	Lymph node	1	4	—	4
Mucosal disease	Lymph node	1	2	—	2
Mucosal disease	Saliva	1	2	—	2
Chicken	Embryo	2	6	3*	3
Skunk	Lymph node	1	2	—	2
Totals		13	30	4	26

* A possibility existed of contamination from active LEA-1 infection in an adjacent stall.

onstrate an enhancing effect on listeriosis in test sheep, although suggestive signs developed in 1 sheep receiving material L65-2. Material (M150) from a steer in which mucosal disease was tentatively diagnosed was without effect.

Material (L207-3) collected from a calf which died late in a herd epizootic of mucosal disease showed no evidence of enhancing listeriosis in sheep, even though material collected from a calf which died earlier in the same epizootic clearly demonstrated an enhancing effect.

The isolation of a second listeriosis-enhancing agent (LEA-2) from a flock of sheep soon after 1 clinical case developed in the flock suggests that such enhancing factors play a part in the naturally occurring listeriosis of sheep. Material LEA-2 was obtained from the blood of lambs with elevated temperatures but no clinical signs of disease. Blood from these same lambs obtained one month later did not contain the enhancing agent.

A summary of all attempts to demonstrate enhancement of listeriosis in sheep with materials from different sources (table 2) includes some attempts not previously mentioned. The enhancing effect has twice been obtained with material from sheep in two natural outbreaks of listeriosis, as well as mucosal disease of cattle. In 11 trials, made with these materials, 25 of 29 sheep (86%) developed clinical listeriosis and the organism was isolated from the brains of the affected animals. For comparison, in 13 trials using 11 different materials, which apparently were devoid of enhancing effect, only 4 of 30 sheep developed listeriosis. Of the 4 sheep, 3 had been given normal chicken embryo together with Listeria; however, 2 of the 3 were kept in a stall adjacent to a sheep which had received active LEA-1 material. The result with normal chicken embryo material may, therefore, be questioned and the possibility of air-borne transmission of LEA-1 considered.

Listeria alone were given intranasally in three trials with 4 sheep each. In one trial, listeriosis did not develop. In a second trial, 2 affected animals, both of which had a chronic pneumonia at the time of exposure, developed signs in 28 and 52 days; the organism was isolated from the brain of both at necropsy (36 days and 53 days after exposure) and there was histological evidence of listeriosis in the brain stem of both. In the third trial, signs developed in 1 sheep 23 days after exposure but were not progressive. The animal was killed 43 days after exposure and, while the organism could not be isolated from any tissues, there were definite lesions of listeriosis in the brain stem. We must, therefore, recognize that 3 of the 12 sheep developed listeriosis following intranasal exposure with Listeria alone. We have no satisfactory explanation for the different results obtained in the group receiving Listeria plus materials devoid of an enhancing agent.

Although an enhancing effect was obtained with materials from both cattle with mucosal disease and sheep with elevated temperatures during an outbreak of listeriosis, there is no reason to believe that the agents are identical. Preliminary work suggests that mucosal disease material which will enhance listeriosis in sheep has a cytopathogenic effect on tissue cultures of bovine kidney cells; LEA-1 material[1] seems to have no such effect.

Relatively little is known about a presumed pathogenic agent in mucosal disease material. Most workers[8-12] report only a mild reaction in calves receiving material from natural cases of the disease. One calf which received material ISC-1 after it had been passed through a sheep developed clinical listeriosis and the organism was isolated from the calf. This animal is of particular interest since the enhancing agent was present in its blood ten days after exposure but not demonstrable in lymph node material 21 days later. An enhancing effect was demonstrable with intestinal parasites of this calf. However, further trials are necessary to establish the enhancing effect of such parasites.

The mode of action of the enhancing materials in pathogenesis of experimental listeriosis is not clear. With intranasal exposure, both enhancing agent and Listeria may pass through the cribriform plate directly to the brain; however, the meninges and olfactory bulb usually fail to yield Listeria on culture, whereas the area of the brain stem almost regularly contains Listeria. Well-developed lesions are found in the medulla oblongata and even the lumbar portion of the spinal cord of some sheep which die a few days after intravenous and intranasal exposure. These lesions are often as well developed as those in sheep which die three weeks or more after intranasal exposure alone. This sug-

gests that the extent of the lesion is not necessarily related to a long incubation period. Often, the infiltrative lesions are adjacent to the neural canal, suggesting that the enhancing agent and Listeria might have gained access with the cerebrospinal fluid. The early development of lesions after intravenous exposure would support such an idea. The residence of Listeria and enhancing agent during the long (2- to 3-week) incubation period after intranasal exposure remains unknown. We need more information on the distribution of Listeria and the enhancing agent in the experimental animal. To promote work in this area, a simplified method for demonstration of the enhancing agent is desirable.

Summary

Material from cattle affected with mucosal disease has demonstrated an enhancing effect on experimental listeriosis in sheep when these materials are given together with *Listeria monocytogenes*. Intranasal exposure of sheep is a critical test for the presence of the enhancing factor. A combined intravenous and intranasal exposure of sheep is a critical test for the absence of such an enhancing factor.

The listeriosis-enhancing factor was again demonstrated in an epizootic of listeriosis in sheep. It was not present in the blood of the same sheep one month later.

The listeriosis-enhancing factor in material from mucosal disease of cattle is probably different from that found in outbreaks of listeriosis in sheep. Various aspects of current information are discussed.

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Studies on Bluetongue. III. The Development of a Modified Live Virus Vaccine Employing American Strains of Bluetongue Virus

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THE DEVELOPMENT of immunological measures for the control of bluetongue has been entirely due to the efforts of workers in South Africa, where bluetongue has been a major disease since its recognition during the latter part of the nineteenth century. Spreull,[1] as early as 1902, demonstrated that sheep could be protected against bluetongue by the simultaneous inoculation of hyperimmune sheep serum and virulent virus. Shortly thereafter, Theiler [2] found that immunity could be produced also by the injection of virus that had been passaged through susceptible sheep. Concluding that the virus had thus become attenuated, he developed a method of immunization, based on the use of viable virus, which was introduced in 1907.[3] This method, with minor modifications, was employed throughout South Africa for the next 40 years and was widely acclaimed as making possible the raising of sheep in otherwise unfavorable areas.

The rather remarkable phenomenon of a virus becoming attenuated by serial passage through members of the natural host species prompted du Toit,[4] in 1928, to repeat the work of Theiler. His studies fully confirmed the earlier findings.

Despite the wide acceptance and generally successful use of the vaccine in South Africa, it nevertheless had two major defects: it occasionally produced severe reactions that simulated bluetongue; in other instances, it failed to confer an adequate immunity. While these shortcomings had been recognized since the vaccine's introduction, they assumed major importance with the increased value of the individual animal as the price of wool advanced, with the more extensive use of the vaccine and, to some extent, with the breeding of hypersusceptible English mutton breeds for the production of high quality fat lambs.

In view of these developments, Neitz [5] reinvestigated the problem of bluetongue and its control in South Africa. His work resulted in two extremely significant findings:

1) The sheep-passaged virus was not attenuated in the strict sense of the word, although it obviously lost some of its original virulence. Since it was originally a strain of low virulence, it was believed that the conditions under which it was maintained in sheep merely served to accentuate this characteristic. Under adverse conditions, particularly in areas where the disease was not enzootic, this virus was capable of causing clinical bluetongue but this occurred less frequently in enzootic areas where immunity was usually established by vaccination, by natural infection, and by the lambs acquiring a degree of passive immunity from the immune dams.

2) Even more important, there are several antigenic strains of bluetongue virus. While all possessed variable degrees of antigenic similarity, the immunity resulting from any one was insufficient to provide complete cross protection. The existence of antigenically heterologous strains rather than the failure of the vaccine to induce a durable immunity was, therefore, the factor responsible for bluetongue occurring in vaccinated flocks.

The dilemma occasioned by these findings was soon resolved by Alexander [6] who succeeded in propagating the bluetongue virus in chicken embryos. In this host, the virus readily became attenuated and conferred a solid immunity unaccompanied by any of the adverse reactions associated with the blood-virus vaccine.[7] Four strains of virus that, on the basis of their antigenic structure, appeared to offer wide pro-

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The authors are greatly indebted to Dr. R. A. Alexander, director of veterinary services for the Union of South Africa, for making available the technical details of the vaccine production methods used at Onderstepoort and for other advice and recommendations, all of which played a major part in the development of the vaccine described herein.

tective coverage, were selected for the development of a polyvalent, modified live virus vaccine. A product that gave highly satisfactory results was developed, entirely supplanting the one introduced by Theiler. This vaccine was dispensed originally in liquid form and, later, as a freeze-dried product.[8]

The discovery, in 1953, that bluetongue was present in the United States,[9] either as an indigenous or a recently introduced disease, resulted in an immediate demand for a satisfactory immunizing product. The proposal that the strains of virus used for vaccine production in South Africa be employed was overruled on the basis of risk. It was felt that sheep in America might be more susceptible to bluetongue than those in South Africa, and that new strains of the virus might thus become established here. Only American strains of the virus were used, therefore, in preparing a modified live virus vaccine according to the method used at Onderstepoort. Attempts to attenuate the virus by serial passage in chicken embryos were the first step toward this objective.

EXPERIMENTAL PROCEDURE

Attenuation of the Virus.—Virus Strains.—When this work was begun, the only American strains of bluetongue virus that had been adapted to propagate in chicken embryos and had definitely been identified were the two that had been isolated from sheep in California.[10] These were designated as strain 8 and strain 11. However, since only preliminary immunological studies had been conducted on these strains, it was considered advisable to work with both in case they might be found to differ immunologically.

Propagation of the Virus.—Fertile hen eggs that had been preincubated for eight days at 37.6 C. were used throughout. A minimum of six eggs was used for each serial passage. Each egg was inoculated by the yolk sac route with 0.2 ml. of a 10^{-3} suspension of chicken embryo from the previous passage. When delays occurred between harvesting and subpassage, the inoculums were held at 4.0 C. as a 50 per cent embryo suspension.

In preparing the inoculum, the embryos were at first decapitated and a representative portion of each was pooled and ground, without diluent, in a glass TenBroeck grinder. A 10 per cent (by volume) suspension of embryo was then prepared in fresh beef heart infusion broth. In the later subpassages, the suspensions were prepared from the entire embryo, and the appropriate dilution was made on a volumetric basis in M/50 phosphate buffer at pH 7.2 to 7.4. The inoculated eggs were incubated in a forced-draught egg incubator, thermostatically controlled at 33.6 C.

Eggs containing dead embryos within 24 hours after inoculation were discarded. Embryos dying on the second and third days following inoculation were harvested. The remaining embryos, both living and dead, were harvested on the fourth day and pooled with the previous two days' harvest. From the pool, a suspension was prepared and used to inoculate eggs of the next virus generation series, as described above.

Titration of the Virus.—In titrating the virus, embryos were harvested and pooled as already described, and minced without diluent, in a cold Waring blendor. A portion of the emulsion was then accurately weighed and a 50 per cent suspension prepared in M/50 phosphate buffer. From this suspension, a dilution of 10^{-1} was prepared in the buffer diluent. Serial decimal dilutions from 10^{-2} through 10^{-7} were then prepared in the same diluent. Fertile hen eggs that had been preincubated for eight days were inoculated by the yolk sac route, each egg receiving 0.2 ml. of inoculum. A minimum of five eggs was used for each dilution of virus. After inoculation, the eggs were incubated at 33.6 C. in a forced-draught egg incubator.

The eggs were candled twice daily and the number of deaths recorded. Any eggs found to contain dead embryos within 24 hours after inoculation were discarded. The $l.d._{50}$ titer of the virus was calculated according to the method of Reed and Muench [11] on the basis of the number of remaining embryos that died by the end of the sixth day of incubation.

Dried preparations of the chicken embryo-propagated virus were reconstituted with sterile distilled water or M/50 phosphate buffer to the original volume of the 50 per cent suspension of wet embryo and titrated as described above.

Tests for Attenuation of the Virus.—To determine whether the virus was becoming attenuated, black-faced, crossbred sheep from 9 to 12 months of age were inoculated periodically with each of the egg-adapted virus strains. These inoculations were made alternately with each strain after every fifth consecutive embryo transfer (table 1). The inoculum consisted of 2.0 ml., given subcutaneously, of a 10 or 20 per cent suspension of ground embryo in either allantoic fluid harvested from the corresponding eggs, fresh beef heart infusion broth, or M/50 phosphate buffer.

Temperatures of the injected animals were recorded twice daily for the next 15 days, and examinations were made once daily for clinical evidence of bluetongue—in particular for the development of hyperemia of the buccal mucosa, followed by excoriation of the gums, tongue, and dental pad, and for the presence of pododermatitis. The test sheep were later tested for immunity to bluetongue by subcutaneous challenge with virulent homologous virus in the form of sheep blood in an equal volume of OCG* preservative solution.

* An anticoagulant preservative solution consisting of 5 Gm. of potassium oxalate, 5 Gm. of phenol, 500 ml. of distilled water, and 500 ml. of glycerin.

Each animal was inoculated with 2.0 ml. of challenge material. Temperatures were again recorded and physical examinations were made over the subsequent 15-day period.

Procedure and Results

Attenuation of the Virus.—Both virus strains were readily propagated in fertile hen eggs by yolk sac inoculation. An effort was made to titrate each strain at every fifth, consecutive egg passage level, but this was not always possible.

With each strain, the concentration of virus apparently reached a maximum level by the fifteenth consecutive egg transfer (table 1). Titrations made at closely spaced intervals in the early passages might have shown that this level was attained much earlier.

In the early egg passages, both strains produced clinical bluetongue in sheep as indicated by depression, fever, and the development of swelling, hyperemia, and occasionally excoriation of the buccal mucosa. Because of the pigmentation of the hooves, however, any pododermatitis that might have been present could not be detected. With continued serial egg passage, both strains produced increasingly milder clinical symptoms in sheep until, by the thirtieth passage level in the case of strain 11, and the 35th in the case of strain 8, hyperemia and mouth lesions were not produced by either strain. However, a sharp febrile response of short duration, accompanied sometimes by depression and inappetence, occurred between the sixth and eighth day after inoculation. All sheep used for the virus testing were immune on challenge, showing that the earlier reactions of these animals were due to inoculation with the egg-adapted virus. Because of the fever and depression that occurred, ten additional egg passages were made and the virus was again tested in sheep.

In view of the fact that the vaccine would be prepared from virus at approximately these egg passage levels, and since it was apparent that, under field conditions, the vaccine virus could be transmitted from vaccinated to unvaccinated animals by biting insects, it was felt advisable to determine whether the modified virus would revert to the virulent form on serial passage in sheep. Accordingly, blood samples were taken on the seventh, eighth, and ninth days after the sheep were inoculated with strains 8 and 11 at the forty-fifth and fortieth egg passage levels, respectively; this blood was pooled, and 10.0 ml. was immediately subinoculated into additional sheep, using 5 animals for each strain. A second subpassage was made in the same manner. Temperatures and clinical observations were recorded as previously described.

Two subpassages in sheep of the egg-adapted virus failed to enhance its virulence (table 1). On the contrary, serial passage seemed to make the virus milder for sheep, judging from the decreasing mild febrile reaction produced.

The availability of 30 bluetongue-immune sheep from this experiment, 15 to

TABLE 1—Embryo Lethal Dose$_{50}$ Titer, Infectivity for Sheep, and Immunogenicity of Strains (8 and 11) of Bluetongue Virus at Various Egg Passage Levels

Egg passage level	Titer* (chicken embryo l.d.$_{50}$) of strains		Reaction produced in sheep by strains		Response to homologous challenge with strains	
	8	11	8	11	8	11
	Wet Embryo Virus					
4	NT	4.25	†4/4 (8)^x	NT	†4/4 immune	NT
10	Void	5.0	NT	2/2 (8)	NT	2/2 immune
15	5.17	5.0	2/2 (mo)^x	NT	2/2 immune	NT
20	5.23	Void	NT	1/1 (mo)	NT	NT
25	5.22	NT	1/1 (mi)^x	NT	1/1 immune	NT
30	NT	6.0	NT	2/3 (f)	NT	3/3 immune
35	4.85	5.85	2/3 (f)^x	NT	3/3 immune	NT
40	5.3	NT	NT	1/5f–1/5^{81x}f–0/5^{82x}	‡ (*See* table 2)	
45	NT	NT	1/5f–2/5^{81}f–0/5^{82}	NT	(*See* table 2)	
	Dried Embryo Virus					
52	NT	5.68	(*See* table 3)		(*See* table 3)	
57	6.0	NT	(*See* table 3)		(*See* table 3)	

* Expressed as log of reciprocal of dilution. † Number injected/number reacting. ‡ Number challenged/number immune.

NT = not tested; (8)^x = severe; (mo) = moderate; (mi) = mild; (f), f = fever only (not less than 106 F.); 81, 82 = subpassage 1, subpassage 2.

each of the two strains, presented a singular opportunity to determine the immunological relationships of the two virus strains. Accordingly, 9 sheep in each immune group were challenged with strain 8 virus and the remainder with strain 11. The results (table 2) provide almost conclusive evidence that strains 8 and 11 are immunologically identical.

Meanwhile, 10 additional egg passages of each strain were made as a precautionary measure. It was then felt that in each case the agent was sufficiently modified to proceed with the production of a test batch of vaccine.

Vaccine Studies.—Cultures of strain 8 virus at the fifty-fifth egg passage level, and of strain 11 at the fiftieth, were forwarded to a commercial laboratory† which undertook to develop the vaccine according to the procedure followed at Onderstepoort. After two additional egg passages, a vacuum-dried product was prepared from chicken embryos infected with each of the two strains of virus. These preparations were forwarded to the School of Veterinary Medicine at Davis for safety tests and potency evaluations in sheep.

In vaccinating sheep against bluetongue in South Africa, not less than 500 sheep-vaccinating doses$_{50}$ (s.v.d.$_{50}$) are given to each animal in a single injection of 1.0 ml. This dosage was selected arbitrarily and was determined for each lot of vaccine by egg titrations. Alexander [6] had established earlier, by parallel titrations in sheep and in chicken embryos, that a 1 to 10 quantitative relationship exists between the embryo lethal dose$_{50}$ (e.l.d.$_{50}$) for bluetongue and the s.v.d.$_{50}$. For example, 0.2 ml. of an embryo suspension having a titer of 10^{-6} would contain 1,000,000 e.l.d.$_{50}$ of virus while 2.0 ml. of the material would contain the equivalent number of s.v.d.$_{50}$.

Safety Tests on the Egg-Adapted Strains of Virus.—It was considered advisable to use at least a tenfold larger dose for vaccinating sheep in the United States against bluetongue than is used in South Africa. However, in the preliminary safety tests, double this amount—10,000 s.v.d.$_{50}$—was used to allow a wide margin of safety. Accordingly, the dried preparations were reconstituted and the e.l.d.$_{50}$ of each determined. Both were then appropriately diluted so that each 2.0 ml. contained 10,000 s.v.d.$_{50}$. Sheep were inoculated with 2.0 ml. of either strain, using 5 animals for each. Temperatures were recorded twice daily and the animals were observed for clinical reactions simulating bluetongue. As a further safety test, two subpassages of each strain were made in sheep as described earlier for the liquid embryo material. Twenty-one days later a challenge dose of virulent homologous strain virus was given the animals.

TABLE 2—Reciprocal Cross-Immunity Tests Between the Vaccine Strains (8 and 11) of Bluetongue Virus

No. of sheep	Immune to	Results of challenge with	
		Strain 8	Strain 11
15	Strain 8 (45th egg passage level)	*9/9 immune	6/6 immune
15	Strain 11 (40th egg passage level)	9/9 immune	6/6 immune
		Results of injection with	
		Strain 8	Strain 11
5	Nonimmune	†3/3 infected	2/2 infected

* Number immune/number challenged. † Number reacting/number injected.

With each of the two strains, moderately high temperatures were produced by the dried embryo material and by the first subpassage blood. In only 1 of the 26 animals inoculated was hyperemia of the buccal mucosa detected. The high temperatures recorded for 3 of the 5 contact control animals were unrelated to bluetongue infection since these animals were uniformly susceptible to subsequent challenge with virulent virus.

The results are summarized (table 3).

Although the reactions obtained in this trial were somewhat more severe than those resulting from the inoculation of the liquid embryo material at a ten-passage lower level, the absence of frank clinical signs of bluetongue, the failure of the febrile response to persist beyond 36 hours, and the fact that virulence was not enhanced by serial passage in sheep indicated that the virus could be used with safety as a vaccine.

A large batch of vaccine containing equal amounts of each strain of virus, as determined by titrations in eggs, was prepared in the commercial laboratory for po-

† Viral and Rickettsial Research section, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

tency testing in sheep. If found to possess suitable immunizing properties, it was then to be used on a field-scale trial basis.

Potency Tests of Virus Strains.—It was originally planned to titrate the vaccine in sheep to determine if the 1 to 10 relationship that Alexander found between the s.v.d.$_{50}$ and the c.l.d.$_{50}$ for the South African strains existed in the case of the California strains also. Unfortunately, suitable test sheep were not available at the time, so the dosage for the potency tests had to be calculated on the basis of titrations in eggs.

Forty sheep from a group in which 5 reacted to inoculation with virulent bluetongue virus were each given 2.0 ml. of the vaccine, *i.e.*, 5,000 s.v.d.$_{50}$. Ten uninoculated sheep were added as in-contact control animals. Temperatures were recorded twice daily, and physical examinations were made daily for 15 days after inoculation.

Moderate to high temperatures occasionally resulted, sometimes accompanied by slight degrees of hyperemia and congestion of the buccal mucosa, and by some depression. However, these reactions were not regarded as unduly severe. In fact, some degree of reaction to the vaccination was considered desirable as an indication of an immune response. Since earlier experimentation had established that the blood of sheep manifesting these mild vaccine reactions was incapable of producing clinical bluetongue in susceptible sheep by injection, the vaccine was considered safe for use under field conditions.

Three to four weeks later, 20 of the vaccinated animals and the in-contact controls were each given a challenge dose of virus. The remainder were retained for challenge at periodic intervals to determine the duration of the immunity resulting from vaccination.

Since it had been established earlier that strain 8 and strain 11 virus were immunologically identical, it was considered justifiable to employ only one strain for the challenge inoculations. Strain 8, which had been studied in greater detail and seemed more stable than strain 11, was selected as the challenge virus. Inasmuch as the unmodified virus remains stable when stored in OCG solution as infectious blood, it was planned to prepare a large batch of challenge virus and to titrate it in sheep so that a constant number of sheep-infecting doses could be routinely employed for challenge. Unfortunately, the results of the titration inoculations were inconclusive, so the 2.0-ml. challenge dose used earlier was employed in this experiment.

Temperatures of the animals used for challenge and controls were again recorded for 15 days after inoculation, and the usual physical examinations made.

All the control sheep reacted strongly to the challenge inoculum, whereas all the vaccinated sheep were refractory. The vac-

TABLE 3—Serial Passage in Sheep of Egg-Adapted Strains (8 and 11) of Bluetongue Virus

Original passage inoculum: dried virus (Lederle) (BT–8 at 57th egg passage level; BT–11 at 52nd) 10,000 s.v.d.$_{50}$ per sheep, s.c.*			Subpassage					
			First inoculum: blood from original passage sheep drawn on 7th, 8th, and 9th days postinoculation and pooled; 10 ml. inoculated i.v.† per sheep		Second inoculum: blood of each strain from 1st subpassage sheep drawn on 7th, 8th, and 9th days postinoculation and pooled; 10 ml. inoculated i.v. per sheep		Response to inoculation with virulent virus	
Virus strain	No. of sheep	Results	No. of sheep	Results	No. of sheep	Results	Strain 8	Strain 11
BT–8	5	2—maximum temperature 106.0 F., no hyperemia	5	2—maximum temperature 106.0 F., no hyperemia	3	No temperature over 105.0 F.	All immune	NT‡
BT–11	5	2—maximum temperature 106.8 and 107.3 F., no hyperemia	5	2—maximum temperature 106.0 F.; 1—slight hyperemia	3	No temperature over 105.2 F.	NT	All immune
Contact control sheep	5	2—maximum temperature 106.0 F.; 1—107.6 F., no hyperemia	....	Normal temperatures	....	Normal temperatures	3 infected	2 infected

* s.c. = subcutaneously. † i.v. = intravenously. ‡ NT = not tested.

cine was thus proved capable of inducing an immune response adequate to protect against a massive challenge with virulent virus. Inasmuch as the pathogenicity tests conducted earlier had indicated that the vaccine could be used without danger of causing clinical bluetongue, large-scale field trials were then undertaken, which are described in publication IV of this series.

Keeping Qualities of the Vaccine.—To obtain an indication of the stability of the vaccine under storage of 4.0 C., titrations were carried out periodically on the dried product, which was dispensed in rubber-stoppered vaccine vials under vacuum. The diluent, M/50 phosphate buffer, was held at room temperature. The titration procedure has already been described in the section of this paper entitled, "Titration of the Virus."

The results (fig. 1) show that there was little deterioration of the vaccine when held at 4.0 C. for the times shown. Variations from one titration to another are no greater than can be accounted for by experimental error.

Discussion

For use in the United States, a modified live virus vaccine containing two American strains of bluetongue virus has been developed according to the method employed at Onderstepoort.‡

Although the evidence available thus far indicates that only one immunological type of the bluetongue virus exists in the United States, the two strains were incorporated in the vaccine in the event that they might have slight immunological differences that could not be detected by the method of testing used.

Limited trials reported herein showed that the vaccine is safe in that it does not cause bluetongue, that the virus fails to regain its virulence by serial passage in sheep, and that it confers a solid immunity to virulent virus. Field trials and extensive field use of the vaccine over the past two years have fully confirmed these findings.

An occasional sheep displays some inappetence and depression shortly after vaccination, particularly if the weather is hot and the animals are exposed to direct sunlight. This observation, and the fact that the vaccine has not been used to any extent in young lambs—in which these reactions would be expected to be more severe—might suggest that further attenuation of the vaccine virus is indicated. Since a considerable margin exists between attenuation and overattenuation with consequent loss of antigenicity, additional egg passage of the vaccine strains would undoubtedly provide a product that would cause a less severe vaccine reaction and yet be equal in immunogenicity to that of the

‡ This was accomplished on a cooperative basis by the School of Veterinary Medicine, University of California; Lederle Laboratories Division, American Cyanamid Co.; and the Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D.C.

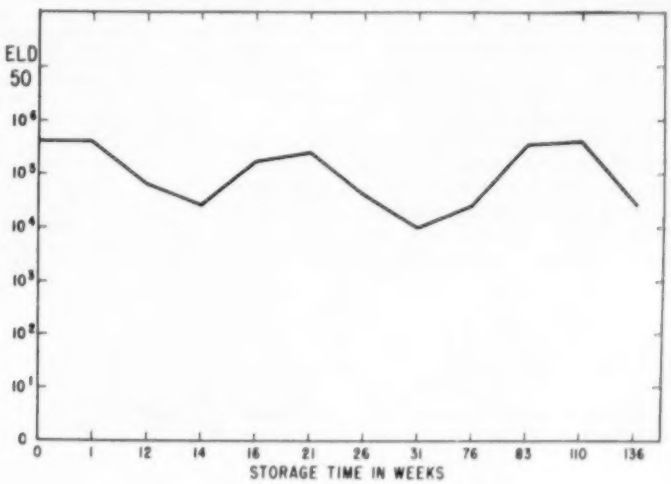

Fig. 1—Stability of the freeze-dried bluetongue vaccine under storage at 4 C.

vaccine strains at their present levels of attenuation. The vaccine issued in South Africa contains four strains of virus at egg passage levels ranging from 72 to 122.

While at Onderstepoort recently as a guest worker, the senior author observed that only Merino sheep, which have non-pigmented buccal mucosa and hooves, are used for studies on vaccine strains of bluetongue virus, and that relatively minor degrees of hyperemia of the buccal mucosa are regarded as significant in evaluating the severity of vaccine reactions. Unfortunately, most of the sheep used in the work reported herein had pigmented buccal mucosa and hooves. It is entirely possible, therefore, that reactions regarded as minimal or not even evident in these animals would have been quite apparent in a white-faced breed such as the Merino.

A feature of the vaccine that is apparently unrelated to the phenomenon of attenuation is that it occasionally seems to cause fetal malformations when ewes are vaccinated during early pregnancy.[12] This is possibly the same phenomenon that has been reported following use of the modified, live virus hog cholera vaccine in pregnant sows.[13] Losses in sheep from this factor could possibly be avoided by vaccinating against bluetongue either before the breeding season or during the later stages of pregnancy.

Summary

With the discovery of bluetongue in the United States, an immediate demand for a vaccine arose. Two American strains of the virus, rather than the South African vaccine strains which might involve risk, were used to develop a modified, live virus vaccine on the basis of the procedure followed at Onderstepoort.

The virus was readily attenuated by serial passage in fertile hen eggs. When the strains were found to produce a negligible clinical and systemic response in sheep, a freeze-dried vaccine was prepared from both strains. Inoculation of the dried produce into sheep showed that: (1) The virus did not regain its virulence by serial passage in the natural host. (2) The virus did not produce unduly severe vaccine reactions in adult sheep. (3) The vaccine protected against a massive challenge with virulent virus.

Large-scale field trials were then carried out with a commercially produced vaccine. These studies are described in publication IV of this series.

Titration of the vaccine, which was stored at 4.0 C., indicated that during a 30-month period the drop in titer was minimal.

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Strain Variation in the Response of Sheep Nematodes to the Action of Phenothiazine. II. Studies on Pure Infections of *Haemonchus Contortus*

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Lexington, Kentucky

PREVIOUS INVESTIGATIONS by the authors [1] indicated consistent and striking differences in the response to phenothiazine treatment between a strain of nematodes (strain B) from a flock with a worm control problem, and a strain (strain A) from a control or normal flock. Observation of a consistently higher threshold for phenothiazine by strain B nematodes suggested the selection or development of drug resistance in this strain from the use of the drug in the flock over a prolonged period.

The present study was undertaken to extend the comparison of these strains and to eliminate the variable factors attending the presence of mixed species by isolating a single species, *Haemonchus contortus*, of each strain. *Haemonchus contortus* was selected not only because it appeared predominant in both strains in the previous study,[1] but also because it has been generally regarded [2,5] as one of the most susceptible of the sheep nematodes to phenothiazine.

MATERIALS AND METHODS

Three experiments were completed in which 14 helminth-free lambs (Cheviot ram crossed with grade Western ewe) were used. The general experimental design and procedures were similar to those outlined in the previous report.[1]

Pure cultures of each strain of *H. contortus* were prepared from natural infections in animals in the respective flocks in the spring of 1955. Mixed nematode larvae were cultured and administered to helminth-free lambs. After patency, these animals were killed, and cultures were prepared from ground-up female *H. contortus* collected from their respective abomasums. Sufficient infective larvae were harvested from the cultures to administer 5,000 to each of the experiment 1 lambs. In succeeding experiments, lambs were infected from the control animals of the preceding experiment. Five thousand infective larvae were used as a standard level of infection.

Only the low level type of phenothiazine administration was studied. Daily doses ranging from 0.1 to 2.0 Gm. for five to seven days were used in the courses of treatment. The same lot of a commercial grade (N.F., green) of phenothiazine was used throughout this study but it was a different lot from that employed in the previous investigation.[1] To insure administration, the daily doses were weighed in gelatin capsules and given by balling gun.

Twenty-four hour fecal collections, for worm egg and larval counts, were made from each animal before, during, and after a course of treatment. The 24-hour fecal collection was thoroughly mixed and two 50.0-Gm. samples were taken. The egg counts were made by a modified Stoll technique,[2] providing dilution factors ranging from five to 100 in the various animals. Four egg counts were made and averaged to compute the 24-hour egg output. The infective (third-stage) larvae were cultured by mixing the other 50.0-Gm. sample with sphagnum moss for storage in pint Mason jars. Routinely, these cultures were put

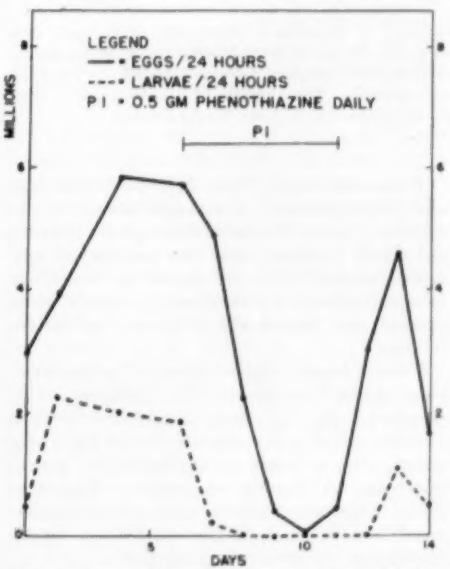

Fig. 1—Egg and larval counts on lamb 58A (experiment 1) infected with strain A Haemonchus contortus.

This investigation is connected with a project of the Kentucky Agricultural Experiment Station, Lexington, and is published with the permission of the director of the Station and the chief of the Animal Disease and Parasite Research Branch, ARS, U. S. Department of Agriculture, Washington, D. C. It is in cooperation with the Animal Disease and Parasite Research Branch, ARS, U.S.D.A.

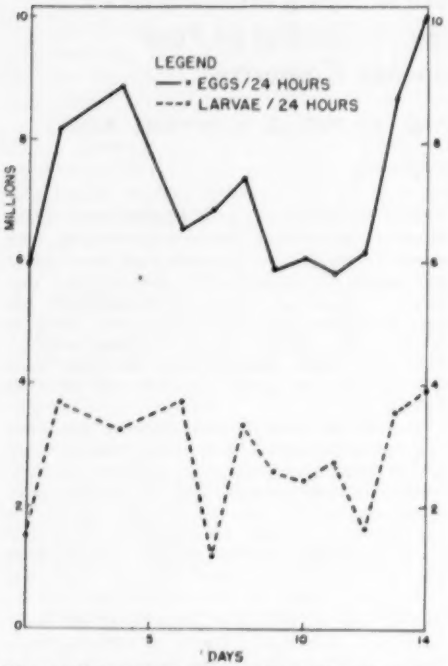

Fig. 2—Egg and larval counts on lamb 58B (experiment 1) infected with strain A Haemonchus contortus.

through a Baermann apparatus after one week and the larvae were estimated by dilution counts with factors ranging from one to 400 in the various animals. The average of six counts was used to calculate the 24-hour larval counts.

RESULTS

Experiment 1.—The 24-hour worm egg and larval counts on 4 lambs are shown in figures 1 to 4. These lambs were 18 weeks old when infected, and the medicated animals weighed 55 to 60 lb. when treatment was initiated. Pretreatment counts were started on the thirty-fifth day after infection.

Five 0.5-Gm. daily doses of phenothiazine, given to lamb 58A infected with strain A (fig. 1), were associated with a marked (83.0 %) reduction in 24-hour egg counts and a complete inhibition of larval development during treatment. Egg and larval counts tended to return to pretreatment levels during the three-day, post-treatment observation period.

Similar treatment of strain B-infected lamb 510A (fig. 3) was associated with practically no change in 24-hour egg counts but with a marked reduction in larval development. The average of the four egg counts during treatment was 90.0 per cent of the pretreatment count average, while the larval development during treatment was only 2.5 per cent of the five-day pretreatment average. Larval development returned to pretreatment levels during the rest period before necropsy.

Egg and larval counts on the untreated animals in this experiment are presented (fig. 2 and 4). At necropsy, at the end of a three-day post-treatment period, the numbers of male and female *H. contortus* recovered from these 4 animals were as follows: 771 and 673 from lamb 58A; 755 and 798 from lamb 58B; 869 and 789 from lamb 510A; and 1,021 and 1,067 from lamb

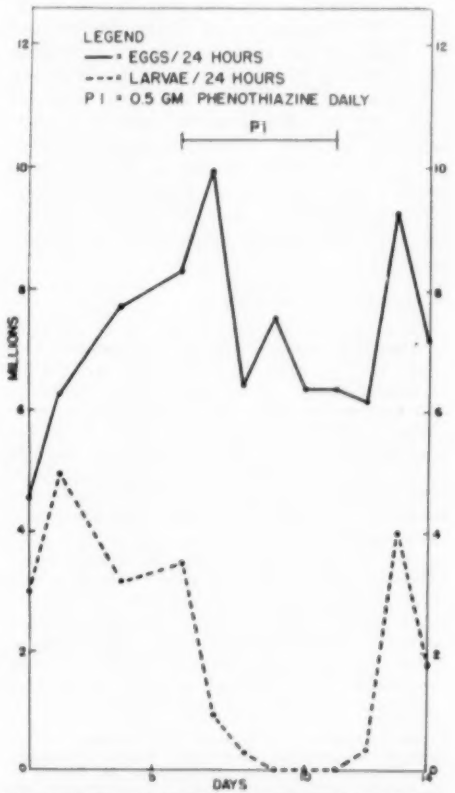

Fig. 3—Egg and larval counts on lamb 510A (experiment 1) infected with strain B Haemonchus contortus.

510B. No other species of nematode was found.

A random sample of 200 female *H. contortus* from the control lambs was examined and classified according to type of vulval process as outlined by Roberts *et al.*[7] From lamb 58B (strain A), 70.0 per cent were sheep strain types A, B, or C and 30.0 per cent were cattle strain types. Those from lamb 510B (strain B) showed 62.5 per cent types A, B, or C and 37.5 per cent cattle strain types.

Experiment 2.—Six lambs were used to provide for 2 treated and 1 control lamb infected with each strain. The lambs were approximately 6 months old when experimentally infected, and the range of body weights of those treated was 53 to 65 lb. Pretreatment observations were initiated on the thirty-third day after the infective larvae were given, and the egg and larval counts during the course of the experiment are presented (fig. 5 to 10).

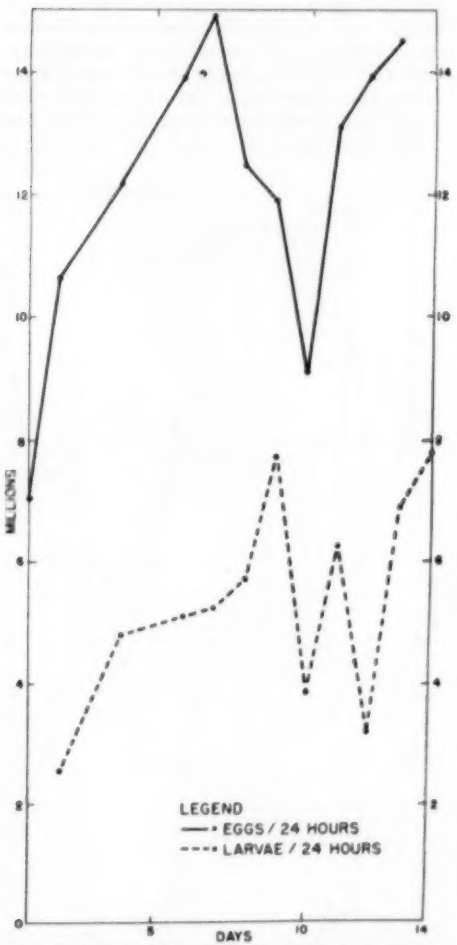

Fig. 4—Egg and larval counts on lamb 510B (experiment 2) infected with strain B Haemonchus contortus.

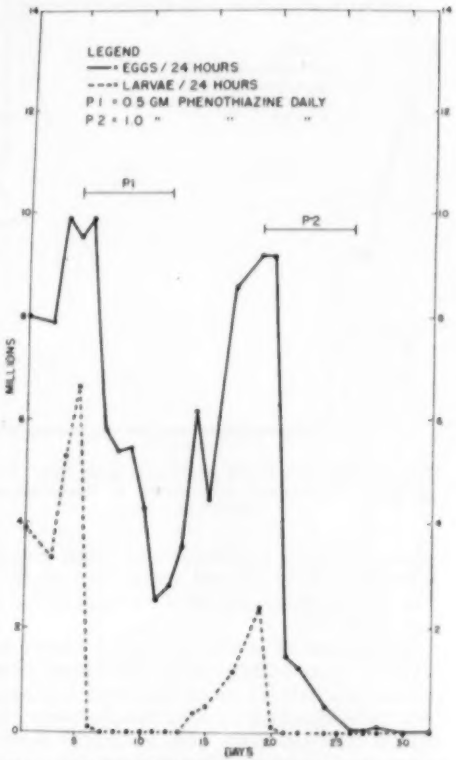

Fig. 5—Egg and larval counts on lamb 50 (experiment 2) infected with strain A Haemonchus contortus.

Treatment of strain A-infected lamb 50 (fig. 5) with 0.5-Gm. daily doses gradually reduced the 24-hour egg count level to one third the pretreatment level and completely inhibited larval development. After a seven-day rest period in which the egg and larval counts tended to recover, a course of treatment at the 1.0-Gm. level not only completely inhibited larval development but depressed the 24-hour egg counts an average of 92.0 per cent. No

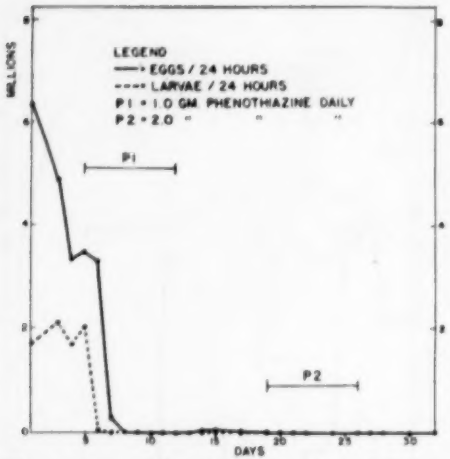

Fig. 6—Egg and larval counts on lamb 56B (experiment 2) infected with strain A Haemonchus contortus.

post-treatment rebound of the egg count level was observed and the return of larval development was at a correspondingly low level.

Treatment of strain A-infected lamb 56B (fig. 6) with 1.0-Gm. daily doses produced effects similar to those observed in lamb 50. Average 24-hour egg counts were reduced 99.0 per cent with no larval development during medication, and low level egg and larval counts after treatment. Subsequent treatment at the 2.0-Gm. daily dose level resulted in markedly reduced egg and larval counts which remained until necropsy of the lamb.

In contrast, treatment of strain B-infected lamb 55 (fig. 7) with a 0.5-Gm. daily dose of phenothiazine failed to produce any essential change in the 24-hour egg counts. Larval counts were reduced an average of 95.0 per cent during treatment, but returned to the pretreatment level during the ensuing rest period. Increasing the dose to 1.0 Gm. daily, after the rest interval, reduced the egg counts only about 25.0 per cent but almost completely stopped larval development. After this second course of treatment, the egg and larval counts returned to the pretreatment levels.

The results of treatment of strain B-infected lamb 57B (fig. 8) with 1.0-Gm. doses were similar to those reported for the preceding lamb. Egg counts were reduced by an average of 20.0 per cent, while inhibition of larval development was practically complete during treatment. During the following rest period, the egg and larval counts returned to the pretreatment levels. Subsequent treatment of this lamb with 2.0-Gm. daily doses depressed the egg counts an average of 66.0 per cent and completely prevented larval development but both returned to pretreatment levels when treatment was discontinued.

The observations on the control lambs (56A and 57A) in this experiment are presented (fig. 9 and 10). At necropsy of both treated and control lambs, on the eighth day after the second course of treatment, the numbers of male and female *H. contortus* recovered were as follows: 843 and 671 from lamb 50; 19 and 7 from lamb 56B; 1,006 and 754 from lamb 56A; 1,267 and 1,069 from lamb 55; 1,338 and 1,072 from lamb 57B; and 1,813 and 1,546

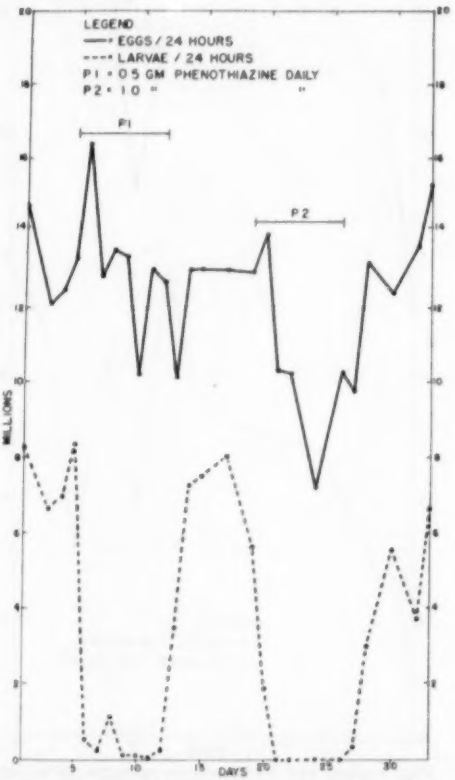

Fig. 7—Egg and larval counts on lamb 55 (experiment 2) infected with strain B Haemonchus contortus.

from lamb 57A. No other species of nematode was recovered from these lambs.

Classification according to type of vulval process of 200 randomly selected *H. contortus* revealed 68.0 per cent types A, B, or C and 32.0 per cent cattle strain types from lamb 56A (strain A), and 60.5 per cent sheep strain types and 39.5 per cent cattle strain types from lamb 57A (strain B).

Experiment 3.—This experiment was designed to observe the effects of lower dose levels, particularly on the strain A parasites. The lambs in this trial were 7½ months old when experimentally infected and weighed from 58 to 75 lb. during treatment. The 24-hour egg and larval counts are shown (fig. 11 to 14). Pretreatment observations were started on the thirty-third day after exposure.

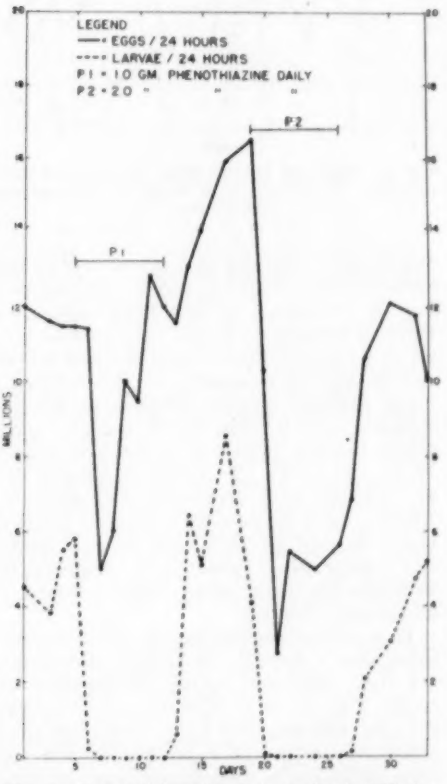

Fig. 8—Egg and larval counts on lamb 57B (experiment 2) infected with strain B Haemonchus contortus.

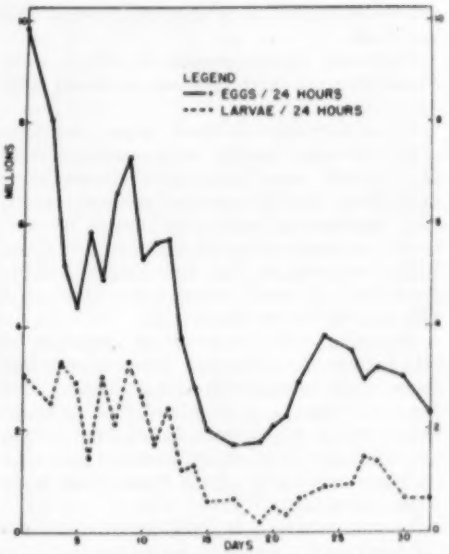

Fig. 9—Egg and larval counts on lamb 56A (experiment 2) infected with strain A Haemonchus contortus.

Two courses of treatment at the 0.25-Gm. daily dose level were administered to strain A-infected lamb 51B (fig. 11). Both courses reduced the average 24-hour egg counts by 66.0 per cent and the larval counts by 99.75 per cent during treatment. The egg and larval counts rebounded after both courses. Subsequent administration of 0.1-Gm. doses to this lamb were associated with an average reduction of egg counts of only 18.0 per cent and larval counts of 97.5 per cent during treatment.

Daily doses of 0.25 Gm. to strain B-infected lamb 513B (fig. 12) resulted in the egg counts during treatment averaging 20.0 per cent below the pretreatment levels and a depression of larval counts during treatment by 84.0 per cent. During the rest period between treatments, this lamb initiated a spontaneous elimination of its worm infection which continued during the repeat therapy at the 0.25-Gm. level until only a light infection remained. This experience was not regarded as a valid test of the drug's activity on strain B.

Control lamb 513A (fig. 14) was substituted for the preceding lamb to test the activity of 0.1-Gm. daily doses on strain B. Average egg counts were 24.0 per cent lower during the treatment period and lar-

val development was depressed only 58.0 per cent.

Egg and larval counts on strain A-infected control lamb 51A are outlined (fig. 13).

Upon necropsy of these lambs, six days after the last course of treatment, only *H. contortus* was found in a complete examination of the gastrointestinal tracts. The numbers of male and female *H. contortus* recovered were as follows: 1,103 and 1,070 from lamb 51B; 997 and 963 from lamb 51A; 1 and 0 from lamb 513B; and 430 and 408 from lamb 513A.

Examination of the vulval processes of 200 female *H. contortus*, taken at random from these worm collections, showed 70.5 per cent types A, B, or C and 29.5 per cent cattle strain types from lamb 51A (strain A); 62.0 per cent sheep strain types and 38.0 per cent cattle strain types from lamb 513A (strain B).

In summarizing the results of the three experiments, the egg and larval counts are consolidated (charts 1 and 2) by expressing the reduction of egg production and larval development at the various dose levels as a ratio of the average egg and larval counts during treatment to the pretreatment average counts. Treatment at the 0.25-Gm. and 0.5-Gm. levels (chart 1) reduced the egg counts of strain A in excess of 60.0 per cent. To obtain a similar degree of egg count depression with strain B, 2.0-Gm. daily doses were required. On this basis, it may be said that four to eight times more phenothiazine was required to produce the same effect against strain B as against strain A.

In a similar comparison of the larval development (chart 2), the lowest dose level, 0.1 Gm., inhibited larval development of strain A in excess of 95.0 per cent, whereas 0.5 Gm. was required for a similar level of inhibition on strain B larvae. If 100 per cent inhibition is used as the point of comparison, doses as low as 0.25 Gm. were effective against strain A, whereas doses of 1.0 Gm. were required against strain B.

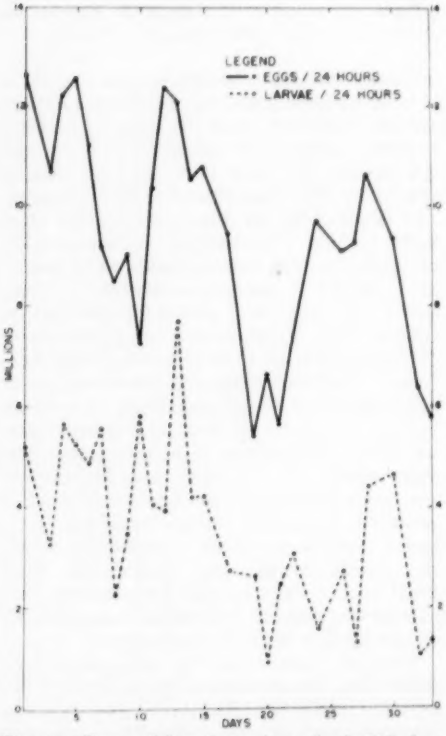

Fig. 10—Egg and larval counts on lamb 57A (experiment 2) infected with strain B Haemonchus contortus.

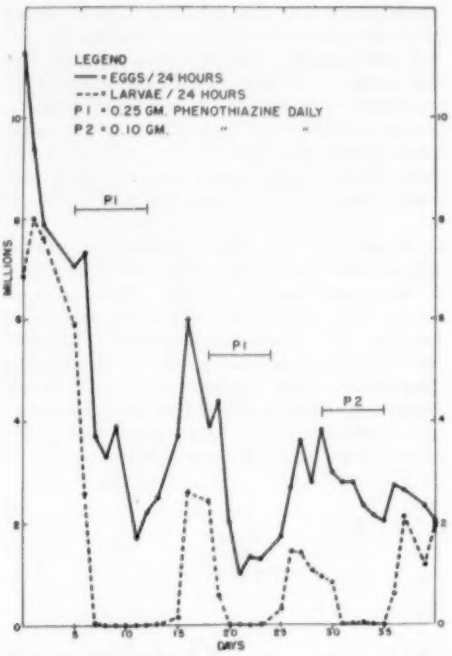

Fig. 11—Egg and larval counts on lamb 51B (experiment 3) infected with strain A Haemonchus contortus.

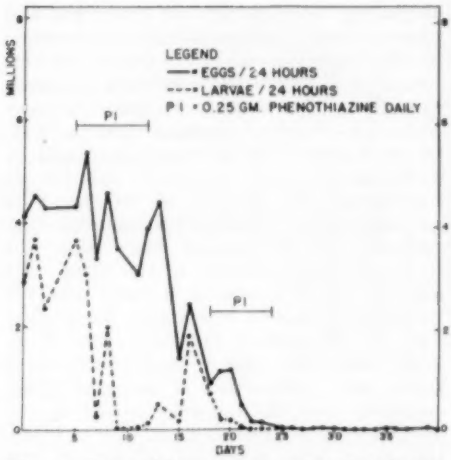

Fig. 12—Egg and larval counts on lamb 513B (experiment 3) infected with strain B Haemonchus contortus.

On this basis, four to five times more phenothiazine was required for the same effect on strain B as on strain A.

DISCUSSION

The observed differences between these two strains of *H. contortus* were remarkably consistent and in close agreement with the earlier studies[1] comparing these strains in the mixed species of nematodes in experimental lambs.

In both strains, there was a predominance of females with sheep-type vulval processes.[7] However, the present strains compared more closely with each other than either did with the characteristic sheep strain reported from Australia.[7] The intermediate position, between *H. contortus* and *Haemonchus placei*, assumed by the strains presents an interesting area for additional investigation, although the observed differences in response to the action of the drug appear too large to be accounted for by this recent species-difference concept.

Strain B parasites not only had a four to eight times higher threshold than those of strain A in the present report, but also appeared to have a higher phenothiazine threshold than strains of *H. contortus* studied by other investigators. Page[6] in working with a strain of *H. contortus* in England found that 0.5-Gm. daily doses of phenothiazine depressed egg counts about 80.0 per cent. This is in close agreement with the effect on the present strain A, whereas four times this level or 2.0-Gm. daily doses were required for a similar depression of strain B egg counts. Gordon[3] reported from Australia that five daily 1.0-Gm. doses were effective against Haemonchus of sheep. This was our finding for strain A, but twice this dose, or 2.0 Gm. for six days, had only a transient and much less marked effect on strain B.

The comparisons in the present study were limited to the low level type of drug administration and would be comparable to the activity obtained from phenothiazine in salt or mineral mixtures. Additional tests are planned to extend the comparison of these strains on the basis of single, therapeutic doses.

Several reports[2,4,6] indicate that the average daily consumption of 0.5 Gm. of phenothiazine in salt mixtures would be effective in controlling the gastrointestinal nematode parasites of sheep. This level of intake has been recorded[10] as an average for sheep in this country. The observa-

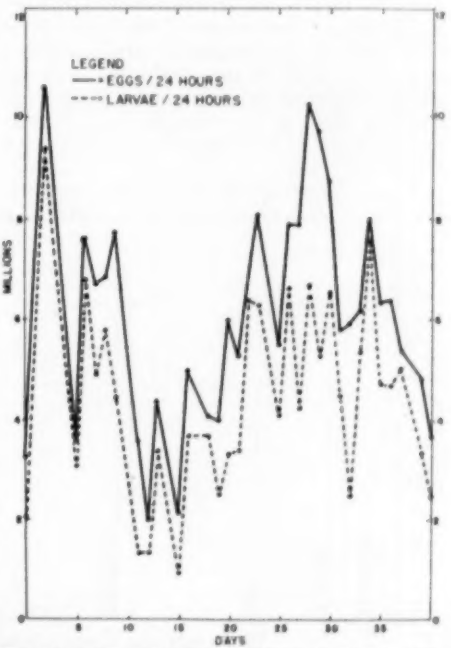

Fig. 13—Egg and larval counts on lamb 51A (experiment 3) infected with strain A Haemonchus contortus.

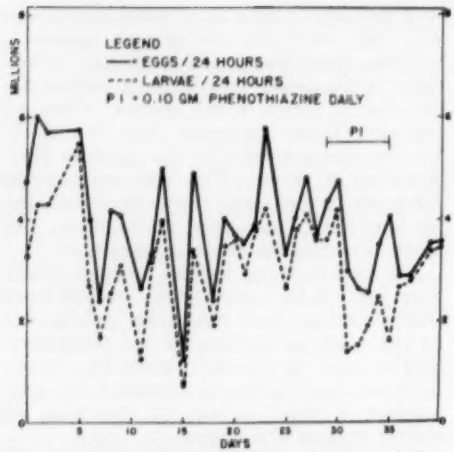

Fig. 14—Egg and larval counts on lamb 513A (experiment 3) infected with strain B Haemonchus contortus.

tions reported here indicate that the 0.5-Gm. daily intake level would effectively control strain A, but not strain B, particularly under flock conditions where many variable factors are encountered. Although a significant limitation of the efficacy of this type of phenothiazine administration is demonstrated by this study, the evidence is not regarded as sufficient to justify excluding the use of phenothiazine in salt or minerals as a prophylactic even with strain B.

These laboratory investigations, and the history of the flock from which strain B originated, indicate that the prolonged and intensive use of phenothiazine has resulted in the selection or development of this strain of *H. contortus* possessing a significant level of resistance to phenothiazine. These observations suggest that a similar or higher level of resistance could be developed under controlled laboratory conditions. The failure of Sinclair [8] to accomplish this with *Trichostrongylus colubriformis* in sheep may have been due to insufficient exposure to the drug. Six generations of worms were used, and the treatment consisted of a single therapeutic dose per generation. In comparison, it appears reasonable that at least two to three times more generations of nematodes had occurred over the ten-year period in the history of the strain B flock. In addition, the drug was used intensively in the flock, both in salt mixtures and in periodic (sometimes monthly) therapeutic drenches over the ten-year period which provided for a more or less continuous contact between the nematodes and the phenothiazine.

The existence or eventual appearance of strains of nematodes possessing equal or greater tolerance to phenothiazine than strain B is also suggested. Records of the department's diagnostic section include an increasing number of cases during the summer of 1955 in which there was evidence of the lack of effectiveness of phenothiazine under field conditions. The present investigations are limited in scope and additional controlled studies are needed.

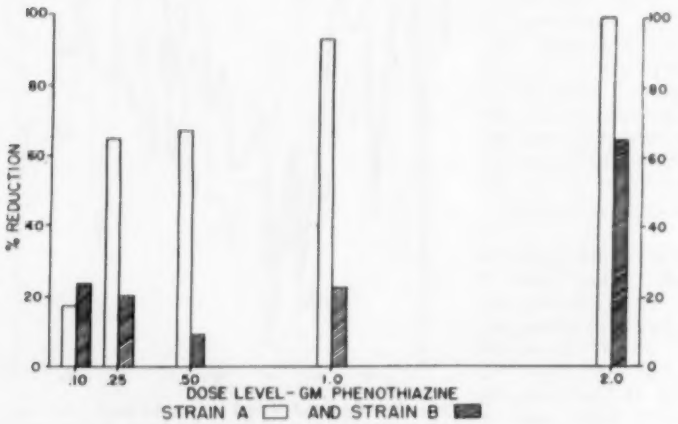

Chart 1—Reduction of egg production during treatment of Haemonchus contortus in sheep.

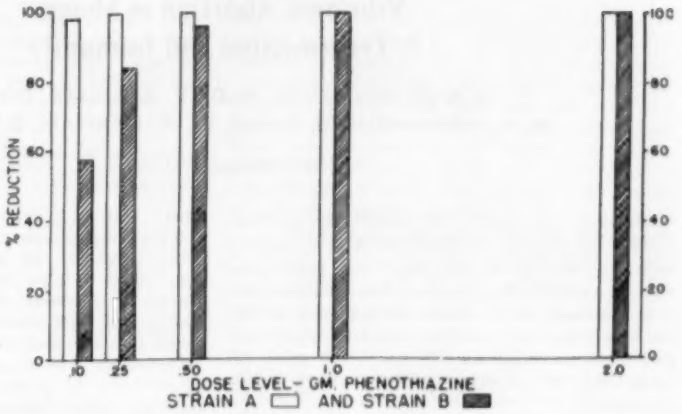

Chart 2—Reduction of larval development during treatment of Haemonchus contortus in sheep.

However, until otherwise established, strain B is regarded as exceptional, and the several other factors that may account for an apparent failure or lack of effectiveness of any anthelmintic should not be overlooked in evaluating an apparent failure or lack of effectiveness of phenothiazine.

SUMMARY

The response of two strains of *Haemonchus contortus* to the action of small daily doses of phenothiazine presented striking differences that agreed very closely with previous findings by the authors. Strain B, from a flock with a worm control problem, not only had a four to eight times higher threshold than the control strain A, against 0.1- to 2.0-Gm. doses, but also appeared to have a higher phenothiazine threshold than strains of *H. contortus* studied by other investigators. These investigations, as well as the flock history, indicate that the prolonged and intensive use of phenothiazine has resulted in the selection or development of this strain of *H. contortus* possessing a significant level of resistance to phenothiazine.

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Vibrionic Abortion in Sheep
I. Transmission and Immunity

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VIBRIONIC ABORTION, an acute infectious disease of sheep, is characterized by abortion during advanced stages of gestation and is caused by *Vibrio fetus*. Herds being maintained in sheds or pasture prior to lambing may incur an incidence of abortion as high as 70 per cent and of mortality of aborting ewes as high as 5 per cent.

This study was conducted to determine: (1) the transmissibility of vibriosis by coition and by oral inoculation and (2) the immunity to vibriosis of pregnant ewes which had been previously exposed to the disease.

REVIEW OF LITERATURE

McFadyean and Stockman[6] studied transmission in multiple experiments and produced abortion in 5 ewes by intravenous inoculation with pure culture of *V. fetus*, in 7 ewes by intravenous inoculation of infected lamb tissues, in 8 ewes by oral inoculation with infected lamb tissues, and in 3 ewes by oral inoculation with pure culture of *V. fetus*. Oral inoculation of 1 ewe with bacteria-free tissue filtrate from an aborted lamb did not produce abortion.

Lee and Scrivner[8] induced abortion in 4 ewes by intravenous inoculation with viable pure culture of *V. fetus* and in 1 ewe by oral inoculation with pure viable culture of *V. fetus*. Intravaginal instillation of a viable pure culture of *V. fetus* in 2 pregnant ewes failed to cause abortion. In a second experiment, Lee and Scrivner[4] studied experimental transmission of vibriosis to 11 pregnant ewes with the following treatments and effects: inoculation of 3 ewes orally with pure culture of *V. fetus* resulted in two abortions; inoculation of 4 ewes orally with infected fetal fluid resulted in two abortions; inoculation of 3 ewes intravenously with the pure viable culture of *V. fetus* resulted in two abortions; and 1 ewe inoculated in the conjunctival sac with the pure culture of viable *V. fetus* did not abort.

Ryff and Lee[9] required 2 experimental pregnant ewes to consume water from a contaminated ranch; both ewes lambed normally. Each of the following treatments were given to a single, separate, pregnant ewe without causing abortion: pure viable culture of *V. fetus* given intravenously; pure viable culture of *V. fetus* given orally; and pure viable culture of *V. fetus* instilled into the conjunctival sac.

Lindenstruth *et al.*[5] studied the relationship of susceptibility of pregnant ewes to the period of gestation. Four different pregnant ewes were each fed viable pure culture of *V. fetus* during each month of gestation and vibriosis was produced in the third, fourth, and fifth months. In another experiment, five lots of 4 pregnant ewes per lot were used to determine the relationship of susceptibility to stage of gestation by intraperitoneal inoculation with viable pure culture of *V. fetus*, with one lot of ewes being inoculated at each month of gestation. Vibriosis was established in the animals in the third, fourth, and fifth months of gestation.

Marsh and Tunnicliff[7] fed viable pure cultures of *V. fetus* to 20 pregnant ewes without causing vibriosis. At the time of copulation, 36 ewes were inoculated into the external orifice of the cervix with viable pure culture of *V. fetus*, also without causing the disease.

Bu *et al.*[2] mated 3 ewes before and 7 after intrapreputial inoculation of rams with viable pure culture of *V. fetus*; vibriosis was not established in any of the ewes.

Baker and Stone[1] observed vibrionic abortion in a flock of 80 ewes. Eight ewes were purchased and observed through normal lambing following subsequent mating with normal rams.

Marsh *et al.*[6] studied vibrionic abortion in 22 ewes which aborted one season. The same ewes showed no evidence of vibriosis during subsequent pregnancies.

Wiggins[10] encountered a high incidence of abortion in 1,768 experimental ewes in 1952. Except for the usual culling, the same sheep with replacements were maintained in 1953. The incidence of abortion in 1953 was at the same low level as for the period of 1940–1951.

MATERIALS AND METHODS

Experiment 1 (1955).—a) Ewes.—A group of 152 ewes were arranged in three isolated lots. The animals, 2 years of age and pregnant for the first time, originated from flocks which had no history of vibrionic abortion. The ewes of lots 1 and 3 were bred by clean rams, while the ewes of lot 2

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This work is an integral part of western regional research project (W-27) for the study of vibriosis of sheep, and was supported in part by the Animal Disease and Parasite Research Branch, ARS, U.S.D.A.

were bred by infected rams. Ewes of lot 3 were each inoculated orally with approximately 40 Gm. of fresh, ground tissues from aborted lambs infected with *V. fetus*. The dates of breeding and lambing or abortion were recorded. Inoculation was at the beginning of the fifth month of gestation.

TABLE 1—Transmission of Vibriosis in Sheep in Experiment 1 (1955)

	Ewes			
Lot	Total	Dead lambs	Oral inoculation	Rams
1	61	0	None	Clean
2	68	0	None	Infected
3	23	17	Infected tissue	Clean

All dead lambs were subjected to necropsy and were examined bacteriologically.

b) Rams.—Preputial mucosal secretions from each ram were examined bacteriologically for *V. fetus*. Rams which were used to breed lots 1 and 3 originated from flocks which had no history of vibrionic abortion and contained no evidence of preputial infection with *V. fetus*. Rams used to breed ewes of lot 2 originated from flocks which had a history of vibrionic abortion and contained *V. fetus* in preputial secretions from natural exposure as well as from superimposed intrapreputial inoculation prior to the breeding season.

Experiment 2 (1956).—a) Ewes.—A group of 241 pregnant ewes were arranged in nine isolated lots. All received similar feed and water. Lots 1 through 7 contained 2-year-old primigravid ewes and 3-year-old ewes which were pregnant for the second time. Lots 1 through 7 were equated for sources of ewes. Ewes of lot 4 were bred by rams which were fed infected lamb tissues prior to the breeding season. Ewes of other lots were bred to clean rams.

The ewes of lot 8 were normal and pregnant for the first time. As nonpregnant yearlings they had been fed infected tissues from lambs which had been aborted as a result of vibriosis. Each ewe in lot 9 aborted from vibriosis the previous year.

The cervix from each ewe of lot 9 and from a sample of 5 ewes from each of lots 1 through 7 was examined bacteriologically for *V. fetus* prior to the breeding season. Dates of breeding and lambing or abortion were recorded for each ewe.

b) Rams.—Prior to the breeding season, the preputial mucosa from each ram was examined bacteriologically for *V. fetus*. Only those rams from which *V. fetus* was not isolated were considered clean. The rams which bred ewes of lot 4 were inoculated orally with infected lamb tissues prior to the breeding season.

c) Inoculum.—Tissues used for oral inoculation of several lots of ewes consisted of ground fetal membranes and intestines of lambs which had been aborted from vibriosis in 1956. The infected tissues were obtained from several infected herds. Tissues from some herds were stored for short periods under refrigeration at —50 C., while tissues from other herds were used without storage. Tissues from all sources were combined to form a single supply of inoculum. Each ewe of lots 1, 8, and 9 received approximately 40 Gm. of the infected tissue orally. Each ewe in lot 2 was given, orally, approximately 50 ml. of filtrate of the infected tissue.

The culture of *V. fetus* for oral inoculation of ewes of lot 3 was isolated from an aborted lamb approximately ten days prior to inoculation. Each ewe was given 25 ml. of the first subculture in thiol medium Difco, containing approximately 300,000,000 organisms per milliliter.

Each ewe of lot 5 was forcibly inoculated orally with approximately 1 gal. of a mixture of manure, mud, and water from an uncleaned pen which contained aborting ewes in 1955.

Each ewe of lot 7 was inoculated orally with approximately 50 Gm. of ground *V. fetus*-infected calf tissues which had been stored under deep refrigeration for a short period.

All oral inoculations of pregnant ewes were made on the same date at the beginning of the fifth month of gestation.

d) Lambs.—Aborted lambs, stillborn lambs, and lambs which died within two days following birth were considered as dead lambs. A gross and bacteriological examination was made of each dead lamb. The number of dead lambs for the lots of ewes was subjected to *chi* square statistical analysis.

TABLE 2—Vibriosis in Sheep in Experiment 2 (1956)

	Ewes				
Lot	Status	No.	Dead lambs	Oral inoculation	Rams
		1) TRANSMISSION			
1	Normal	21	16	Infected tissue	Clean
2	Normal	21	2	Infected tissue filtrate	Clean
3	Normal	23	3	Culture *V. fetus*	Clean
4	Normal	22	3	None	Infected orally
5	Normal	21	2	Manure	Clean
6	Normal	25	2	None	Clean
7	Normal	20	1	Infected cattle tissue	Clean
		2) IMMUNIZATION			
8	Fed infected tissue (1955)	44	3	Infected tissue	Clean
9	Aborted 1955	44	4	Infected tissue	Clean

RESULTS

Experiment 1.—No abortions occurred in lots 1 or 2, while of the 23 ewes of lot 3, 17 (73.9 %) aborted (table 1). *Chi* square statistical analysis indicated a high significant difference ($P < .01$) between the lots for the number of ewes with dead lambs.

Experiment 2.—The number of ewes with dead lambs for each lot is presented (table 2). *Chi* square statistical analysis showed a high significant difference ($P < .01$) between lots for the number of ewes with dead lambs. Excluding lot 1, *chi* square statistical analysis showed no significant difference ($P > .05$) between lots 2 through 9 for the number of ewes with dead lambs.

Vibrio fetus was isolated from many aborted lambs of lot 1; from 1 aborted lamb from each of lots 2, 3, 4, and 7; and from 2 aborted lambs from each of lots 8 and 9.

Interval from Inoculation to Abortion.—For experiments 1 and 2 combined, the number of days between inoculation and abortion varied from seven to 25 with a mean of 13.2 ± 4.42.

DISCUSSION

Oral inoculation with infected fetal tissues appears to be a reliable method of reproducing vibrionic abortion in ewes during the last month of gestation. This method of producing the disease suggests that contamination of feed and water is an important factor in spreading the disease. Under confinement, aborting ewes have ample opportunity to expose other ewes by contamination of feed and water with fetal tissue and fluids and with uterine discharges. The exercise of sanitary principles, especially early in an epizootic, should retard or prevent the spreading of the disease to susceptible ewes. Although epizootics may spread as a result of contamination of ingesta, the pathogenesis of infection for the first abortion in a flock is unexplained.

The inability to establish the disease in susceptible ewes by coition with infected rams is at variance with the transmission of bovine vibrionic abortion, a venereal disease.

In experiment 2, the failure of ewes of lot 3 to develop abortions beyond the expected number may be due to rapid diminution of pathogenicity of *V. fetus* during the first cultural transfer or to the absence of an accessory factor which may be present under natural conditions and which may be synergistic with *V. fetus* to constitute the full etiological agent. The presence or absence of a synergistic factor may be determined by inoculation of susceptible ewes with a mixture of culture of *V. fetus* and bacteria-free fluids from infected fetal tissues.

Solid immunity following abortion, as shown in ewes of lot 9 of experiment 2, is in agreement with the consensus from the study of natural epizootics of vibrionic abortion. Under field conditions, however, failure of the disease to recur may have been due to immunity or absence of exposure. The induction of immunity in ewes of lot 8, which were fed infected fetal tissues as nonpregnant yearlings, indicated that immunization can be independent of pregnancy and abortion.

SUMMARY

Two experiments, using 393 ewes, were conducted. In experiment 1, ewes of lots 1 and 3 were bred to clean rams and ewes of lot 2 were bred to infected rams. At the beginning of the fifth month of gestation, each ewe of lot 3 was fed forcibly approximately 40 Gm. of ovine fetal tissue infected with *Vibrio fetus*. No abortions occurred in ewes of lots 1 and 2, while 73.9 per cent of the ewes of lot 3 aborted.

In experiment 2, 241 ewes were arranged to form 9 lots. A lot of normal ewes which was fed ovine fetal tissue infected with *V. fetus* at the beginning of the fifth month of gestation presented a significantly higher rate of abortion than did similar lots of ewes fed either culture of *V. fetus*, bacteria-free infected tissue filtrate, manure from an infected lot, or bovine fetal tissue infected with *V. fetus*.

Ewes which aborted from vibriosis during the previous year, and ewes which were fed infected ovine fetal tissue prior to pregnancy, were immune when challenged by oral inoculation with infected ovine fetal tissue during the fifth month of gestation.

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Physiological and Nutritional Changes in Lambs Infected with the Nematodes, *Haemonchus Contortus, Trichostrongylus Colubriformis,* and *Nematodirus Spathiger*

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NUTRITIONAL and physiological aspects of host-parasite relationships have been investigated only to a limited extent. Most observations concerning the effects of roundworms on their hosts have been made with experimental infections of single species. Little information is available as to the interaction of several nematode species in simultaneous multiple infections.

Stewart [1] found that digestibility of crude protein increased as worm egg counts decreased in lambs with mixed natural nematode infections. When infections were heavy, there was also an impairment of crude fiber digestibility although no effects on digestibility of nitrogen-free or ether extracts were noted.

Shearer and Stewart [2] observed interference with calcium and phosphorus metabolism but no change in the metabolism of sodium and potassium in lambs heavily infected with a mixture of intestinal nematodes.

Andrews [3] studied the effect of pure infections of *Cooperia curticei* on the nutrition of lambs and found no effect on digestibility or absorption of various nutritional components, although there was some effect on the utilization of these components due to an increase in energy metabolism.

Andrews *et al.*,[4] investigating the effects of *Trichostrongylus colubriformis* on the nutrition of lambs, failed to confirm the findings of Stewart [1] and Shearer and Stewart.[2] However, in 1 lamb which died of trichostrongylosis, the apparent digestibility coefficients of all components of the feed, except those of ether extract and crude fiber, were decreased when the vitality of the animal had been reduced.

Franklin *et al.*[5] experimentally infected lambs with *T. colubriformis* and found a lower rate of live-weight increase, lower protein digestion, and poor utilization of calcium and phosphorus. Blood analyses revealed low calcium and phosphorus levels.

A comprehensive review of nutritional host-helminth relationships was prepared by Hunter.[6]

The purpose of this investigation was to determine the effects of heavy mixed infections of three of the more common sheep nematodes on various nutritional and physiological components.

MATERIALS AND METHODS

Eight grade lambs of approximately the same age, raised "parasite free,"[7] were placed in two pens so that 3 wethers and 1 ewe were in the first lot and 2 wethers and 2 ewes in the second lot. The pens were constructed of concrete walls and floors with a central drain in each. No bedding was used but each pen was cleaned by washing daily.

The ration was a pelleted feed consisting of 40 per cent green alfalfa meal, 49 per cent ground yellow corn, 10 per cent molasses, and 1 per cent salt with trace minerals. Before pelleting, 10 lb. of chromium oxide was uniformly mixed with each ton of feed as a base for digestibility trials. Daily feed and water consumption records were kept. Water containers were such that, when full, the exposed surface areas were approximately the same.

Fecal material from each of the lambs was checked daily for the presence of worm eggs. All fecal matter from the wethers was gathered and analyzed as follows:

1) One aliquot of 10 Gm. was used to check for parasites.

2) One aliquot of 10 Gm. was used to determine the hydrogen ion concentration.

3) The remainder was weighed and dried in an oven at 65 to 70 C. until two successive weighings at 12-hour intervals were the same. Each dried sample was then finely ground and analyzed for crude protein, by a modified Kjeldahl method (using selenium as a catalyst), and crude fiber, by the conventional method. Analyses for chromium oxide were according to Bolin *et al.*[8]

This is a report of cooperative research between the North Dakota Agricultural Experiment Station and the Animal Disease and Parasite Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

From the Department of Veterinary Science (Shumard and Eveleth) and the Department of Animal Nutrition (Bolin), North Dakota Agricultural College, Fargo. The authors express their thanks for the valuable technical aid of D. J. Hanson, C. L. Lohse, and F. Hesse.

Published with the approval of the director, North Dakota Agricultural Experiment Station, Fargo, and the chief of the Animal Disease and Parasite Research Branch, ARS, U.S.D.A.

TABLE 1—Number of Worms Recovered at Necropsy from Experimentally Infected Lambs

Lamb No.	*Haemonchus contortus*	*Trichostrongylus colubriformis*	*Nematodirus spathiger*
53	2,520	31,000	190
60	12,050	20,410	1,060
61	104	39,060	106
71	0	31,600	160

The same analyses described above were carried out on an aliquot of each bag of feed used. All analyses were duplicated and averaged.

Hydrogen ion concentration of the feces was measured by mixing 10 Gm. of feces in 40 ml. of distilled water and making direct readings on a Beckman glass electrode pH meter.

Weights of all animals were taken at approximately weekly intervals. Two blood samples of 5 ml. each were withdrawn daily from the jugular vein of each lamb. One was allowed to clot; the other was mixed with sodium citrate (0.05 ml. of a 10% solution, evaporated to dryness). The serum was removed from the clotted samples and analyses for inorganic phosphorus (modified from Fiske and Subbarow[9]), total protein (Kingsley[10]), albumin (Pillemer and Hutchinson[11]), and the globulin content were calculated. Hematocrit values were determined by the method of Van Allen,[12] blood glucose by a modification of the Folin-Wu[13] method, and hemoglobin by the oxyhemoglobin method.

Three days after the lambs were placed on the experiment, each of the lambs in one lot was given approximately 150,000 infective larvae consisting of 100,000 *T. colubriformis*, 40,000 *Haemonchus contortus*, and 10,000 *Nematodirus spathiger*. All of the infected lambs were given larvae from a single container in which the three species were thoroughly mixed before each dose was withdrawn.

As each lamb died, the contents of the abomasum and the intestinal tract were removed and preserved in 10 per cent formalin. Worm counts were made from this material by a dilution method except when less than 1,000 worms were present at which time they were individually counted.

RESULTS

The number and species of worms recovered at necropsy are shown (table 1). All

TABLE 2—Feed and Water Consumed per Pound of Infected and Control Lambs *

Period following infection (days)	Av. daily consumption			
	Lb. of feed/lb. of lamb		Qt. of water/lb. of lamb	
	Infected	Control	Infected	Control
0 to 5	0.016	0.02	0.02	0.021
5 to 10	0.015	0.019	0.024	0.023
10 to 15	0.012	0.02	0.023	0.024
15 to 20	0.006	0.025	0.02	0.025
20 to 25	0.005	0.025	0.02	0.024
25 to 27	0.001	0.022	0.016	0.024

* Based on average weight for period indicated and total feed and water consumed during the period.

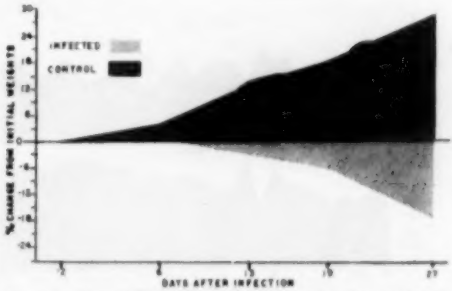

Fig. 2—Average weight changes of lambs infected with nematodes and of their uninfected controls.

4 lambs were susceptible to larvae of *T. colubriformis* but not as uniformly susceptible to the other species. No Haemonchus were found at the necropsy of lamb 71, while lamb 60 harbored five times as many Haemonchus and five times as many Nematodirus as the next most highly infected lamb.

Average daily feed consumption is graphically depicted (fig. 1); that of the

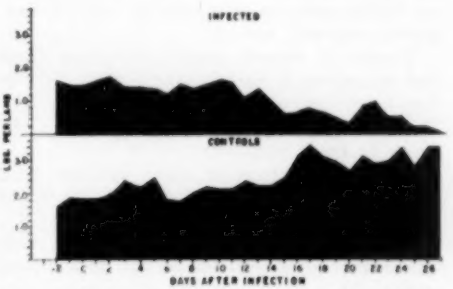

Fig. 1—Average daily feed consumption of lambs infected with nematodes and of their uninfected controls.

infected lambs dropped rapidly beginning 13 days after infection and was reflected in the weight changes (fig. 2). The more rapid decline in weight occurred after 19 days following infection. This was shown in the amount of feed consumed per pound of lamb (table 2).

Apparent average daily water consumption (fig. 3), disregarding evaporation was not as severely affected by the infections as was feed consumption. When computed on a weight basis (table 2), this consumption did not appreciably change until 25 days following infection.

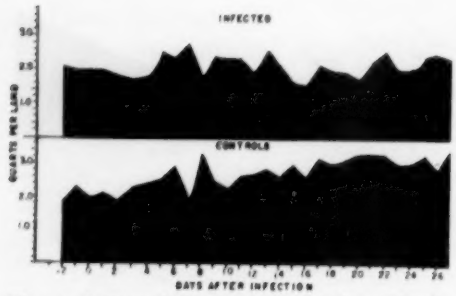

Fig. 3—Average daily water consumption of lambs infected with nematodes and of their uninfected controls.

The percentage of water in the feces of the wether lambs is illustrated (fig. 4). There was more variation in fecal moisture among the infected animals. Initial increases for both infected and control animals can be accounted for by corresponding increases in water consumption. However, the rapid decrease followed by an increase in moisture in the feces of lamb 71 does not correspond to fluctuation in water consumption. This lamb showed no indications at necropsy of having harbored stomach worms.

Lamb 60 passed semiliquid feces from the seventeenth to twentieth days following infection, a period during which Trichostrongylus ova began to appear in the feces in large numbers.

A rapid decline in fecal moisture occurred preceding the death of lambs 53 and 60.

No significant changes in fecal pH took place during the course of the experiment (table 3).

Because of the hemorrhagic conditions produced by clinical infections with *H. contortus*, hemoglobin levels and hematocrit values are usually indicative of the extent of infection. These values fell rapidly in 3 of the infected animals (fig. 5 and 6). Lamb 71 did not respond in this manner, however, indicating that few if any Haemonchus were present. Lamb 61 which harbored only 104 Haemonchus at necropsy responded with severe clinical signs of haemonchosis.

Blood glucose levels (fig. 7) were genarally higher in the infected lambs than in the controls after the thirteenth day following infection. Inorganic serum phosphorus levels in all infected animals (fig. 8) were depressed to their lowest points from ten to 15 days following infection and then recovered somewhat. One of the control lambs (56) exhibited a gradual depression in levels of this element. The relative decrease for inorganic serum phosphorus, comparing the lowest with preinfection levels, ranged from 48 to 82 per cent. The decrease for control lamb 56 was 24 per cent.

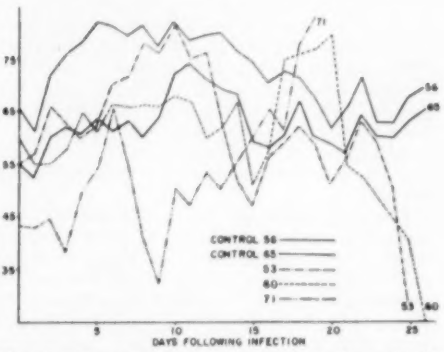

Fig. 4—Percentage of water in feces from lambs infected with nematodes and from their uninfected controls.

One of the more striking effects of the infections was on protein. The globulins increased with a corresponding decrease in albumins in the infected animals (fig. 9). This change seemed to be largely in-

TABLE 3—Fecal pH of Infected and Control Lambs

Lamb No.	Days after infection											
	0	1	3	6	8	11	13	15	17	19	21	25
						Infected						
53	8.0	8.0	8.3	7.4	7.4	7.9	8.0	8.6	7.7	7.9	8.5	7.8
60	8.0	8.1	8.5	8.3	7.6	7.9	8.1	8.3	7.5	7.2	7.9	7.8
71	8.1	8.4	8.3	8.5	7.8	8.3	8.4	8.3	7.3	....	....	....
						Control						
56	7.1	7.7	7.1	6.6	6.7	7.5	7.3	7.3	7.6	8.5	8.4	8.0
65	8.5	8.6	8.3	8.5	8.2	8.7	8.7	8.8	8.5	8.5	8.5	8.5

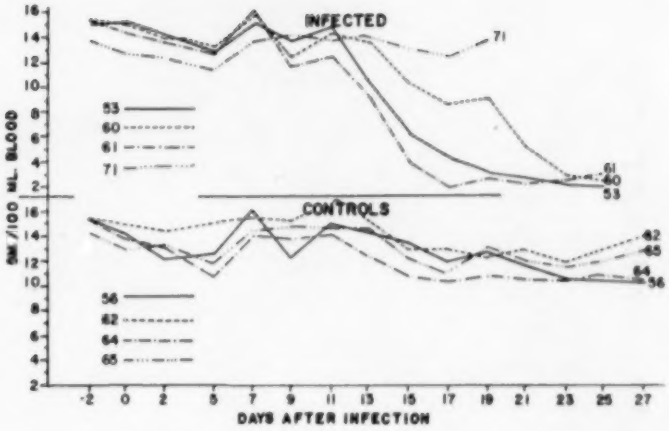

Fig. 5—Blood hemoglobin levels of lambs infected with nematodes and of their uninfected controls.

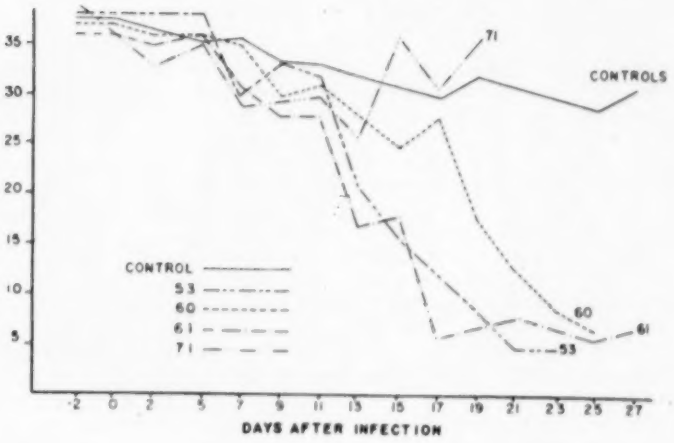

Fig. 6—Hematocrit values of lambs infected with nematodes and of their uninfected controls.

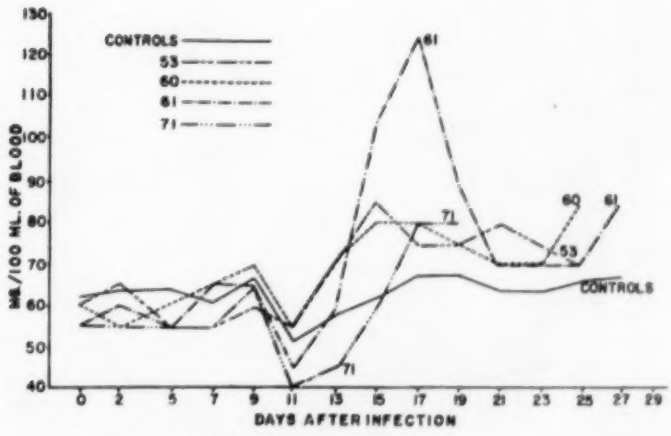

Fig. 7—Blood glucose levels of lambs infected with nematodes and of their uninfected controls.

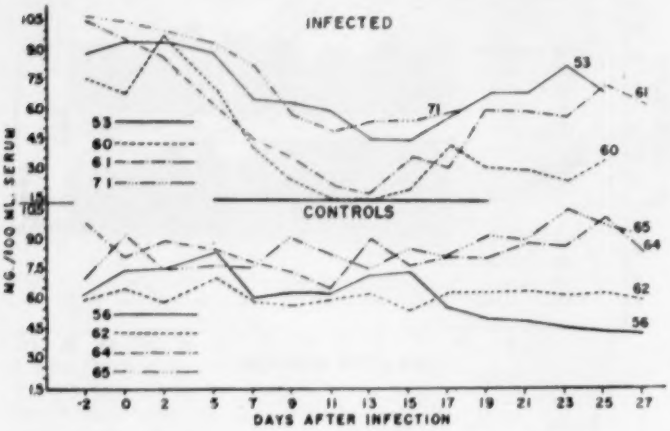

Fig. 8—Inorganic serum phosphorus levels of lambs infected with nematodes and of their uninfected controls.

dependent of fluctuations in total proteins (fig. 10). Depressions in the levels of total serum protein of the infected lambs occurred between the tenth and nineteenth days following infection. Recovery to nearly normal levels occurred in all infected animals a few days preceding death.

The digestibility of protein was interfered with in all of the infected wethers (fig. 11). The first nematode eggs appeared in the feces on the sixteenth day following infection. It was presumed that the presence of these eggs would interfere with quantitative results; therefore, fecal analyses were suspended on this day.

Marked fluctuations in the digestibility of crude fiber occurred in the infected wethers. Other than this variability (fig. 12), no significant changes took place.

DISCUSSION

Variations in the susceptibility of lambs to various species of nematodes have been reported by Clunies Ross,[14] Boughton and Hardy,[15] and Fourie.[16] Lucker,[17] noted a lack of uniformity in susceptibility of 16 young lambs with controlled experimental infections of *H. contortus*. An average of 26.9 per cent of the larvae he administered developed in the lambs and were recovered at necropsy (range, 0.7 to 67.6%). In this study, an average of 9.2 per cent of the Haemonchus larvae developed and were recovered at necropsy (range, 0 to 30.1%);

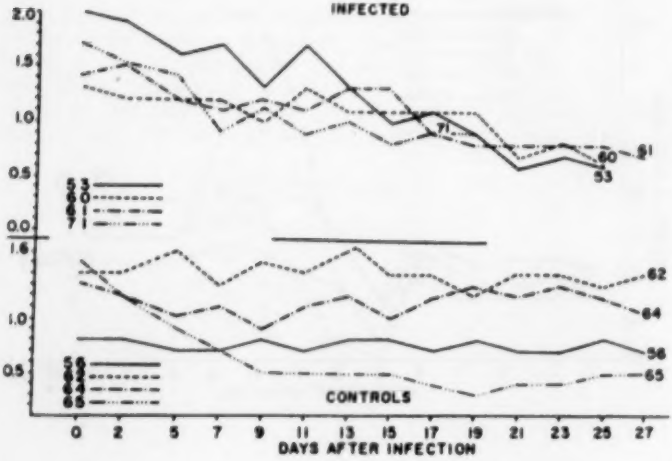

Fig. 9—Serum albumin-globulin ratios of infected and control lambs.

an average of 30.5 per cent of the Trichostrongylus developed and were recovered (range, 20.4 to 39.1%); and an average of only 3.8 per cent of the Nematodirus developed and were recovered (range, 1.1 to 10.6%). The wide variation in susceptibility to *H. contortus* and *N. spathiger*, with a corresponding uniform susceptibility to *T. colubriformis* in lambs of approximately the same age, indicates that the resistance to acquisition of these parasites is probably inherent and that it is also species specific.

The marked resistance to the establishment of *H. contortus* infection by lamb 71 permits a comparison of the effects of this species and *T. colubriformis*, if we assume that the number of Nematodirus present were insignificant. Hemoglobin and hematocrit levels for lamb 71 were comparable to control levels. Therefore, little or no hemorrhage or other effects on hemopoiesis occurred in this lamb. Only 104 stomach worms were recovered from lamb 61. The hemoglobin and hematocrit levels for this lamb were as severely depressed as for lamb 60 which harbored 12,050 of this species. Some of the worms may have passed out of lamb 61 during the debilitated state.

Inorganic serum phosphorus levels were not as depressed in lamb 71 as in the other lambs; however, a significant decrease took place which agrees with the findings of Franklin *et al.*[5] Lambs infected with all three species of worms were more severely affected in depression of total serum protein and in digestibility of protein than lamb 71. This suggests that the effects of the worms are cumulative but not necessarily proportional to the number present.

Infected lambs exhibited a hyperglycemia and a hypophosphotemia. High blood glucose levels occurred despite a decrease in feed consumption. The low phosphorus levels were probably due to both lower feed intake and interference with intestinal absorption as described by Franklin *et al.*[5] Hyperglycemia could, therefore, have resulted from a lack of phosphorus; the available glucose probably could not enter the scheme of glycolysis because of this deficiency. It is possible, however, that gluconeogenesis produced the hyperglycemia due to increased production of

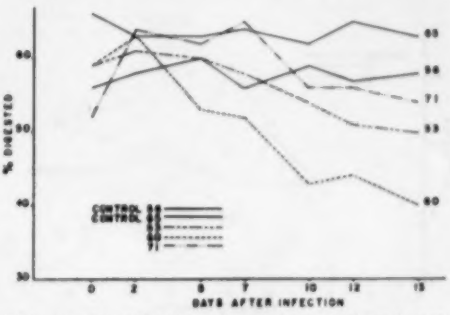

Fig. 11—Digestibility of protein in infected and control lambs.

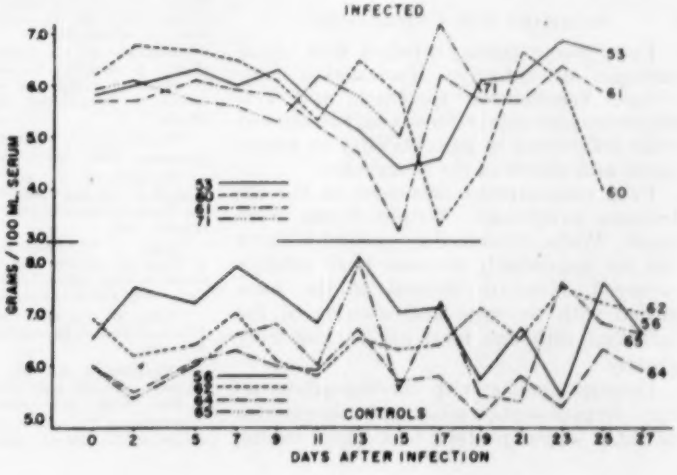

Fig. 10—Total serum protein of infected and control lambs.

glucocorticoid hormone from an adrenal cortex influenced by extreme stress.

Partial recovery of inorganic serum phosphorus occurred when the lambs were in a debilitated state. Energy metabolism at this time was probably slight. The need

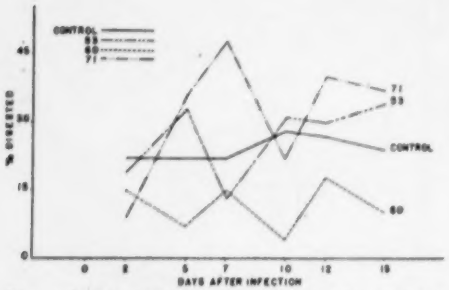

Fig. 12—Digestibility of crude fiber in infected and control lambs.

for phosphorus thus could have been decreased allowing the serum levels to become more concentrated. It is doubtful that these levels resulted from increased absorption from the intestines. A corollary to these assumptions is the fact that serum protein levels also rose during the debilitated state until a time when no feed and little water was being consumed.

Depression in digestibility of protein could have been due to interference with digestive or absorptive processes either by mechanical means or by the influence of exotoxins.

SUMMARY AND CONCLUSIONS

Four young lambs, infected with equal numbers of infective *Haemonchus contortus, Nematodirus spathiger,* and *Trichostrongylus colubriformis* larvae showed wide differences in susceptibility to acquisition and effects of the nematodes.

Feed consumption decreased as the infections progressed. Weight losses were rapid. Water consumed per pound of lamb did not appreciably decrease until debility occurred. Moisture content of the feces varied with the type and severity of the infection, although fecal pH did not vary greatly.

Hyperglycemias with corresponding serum hypophosphotemias and depressions in total serum protein took place in the blood of all infected lambs. Albumin-globulin ratios increased in all infected lambs.

Digestibility of protein gradually diminished while digestibility of crude fiber fluctuated widely as the infections progressed.

Blood hemoglobin and hematocrit values indicated severe haemonchosis in all except 1 infected animal from which no *H. contortus* were recovered upon necropsy.

From the foregoing observations, we conclude that differences in initial susceptibility to acquisition of the species of nematodes used are inherent and probably species specific.

Lambs infected with *H. contortus, T. colubriformis,* and *N. spathiger,* or *T. colubriformis* and *N. spathiger* in the numbers specified in the experiment can not efficiently utilize protein and become hypophosphotemic and hyperglycemic. Lambs infected with *T. colubriformis* and *N. spathiger,* in the numbers indicated, do not exhibit depressions in hemoglobin and red blood cell volumes as do those lambs which are also infected with *H. contortus.*

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Blood Constituents in Suffolk Ewes and Lambs

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IN THE COURSE of a prolonged experiment on the biological effects of I^{131} administered to Suffolk sheep,[1] numerous control values for blood chemistry and hematology were obtained. These data were a prerequisite for determining which, if any, of the blood constituents would reflect an early change due to I^{131} administration. This paper presents a compilation of the determinations of the blood cellular elements, hemoglobin, and serum levels of cholesterol, glucose, inorganic phosphorus, alkaline phosphatase, calcium, creatinine, protein-bound iodine (PBI), and nonprotein nitrogen (NPN) in purebred Suffolk sheep, with the intention of showing the trends and variations occurring in a flock maintained under laboratory conditions.

METHODS

Purebred Suffolk ewes and their 1950 and 1951 offspring were used in the study. In November, 1949, the original ewes were moved from local range country to project quarters where they were bred in January and February of 1950. The reproductive cycle of the ewes and their lambs during various months of the year is shown (table 1).

The animals were confined to concrete-floored laboratories during the entire experiment. Their ration consisted of chopped alfalfa hay and a grain mixture containing (by weight): 8 parts rolled oats, 8 parts ground peas, and 1 part linseed meal. One half to 1 lb. of dried-molasses beet pulp was included in the ration and the animals were given free access to steamed bone meal and salt of low iodine content. For experimental purposes, the original ewes were separated into three groups of 24 each and the lambs into units of about 10 each. Results from each group were pooled. The number of sheep involved in the experiment was diminished gradually by planned slaughter or diversion to other experiments. The values for 1954 (not listed in table 1) were obtained from 4 sheep of each age group from which blood samples were taken at weekly intervals for four months.

At established intervals of about three weeks, blood was taken by jugular puncture, using a 21-gauge needle and evacuated tubes. Heparin was used as an anticoagulant for cellular studies. Erythrocytes were diluted with saline and leukocytes were diluted with 0.1 N HCl for enumeration. Differential leukocyte counts were made on smears stained with Wright's stain and were based on 200 cells. For determination of the packed volume of erythrocytes, Wintrobe's hematocrit tubes were centrifuged at 2,700 r.p.m. for 30 minutes. Hemoglobin concentrations were determined as acid hematin from 1950 through 1952. During the first two years, a precalibrated Lumetron colorimeter was used but, in 1952, whole blood standards based on the iron content, as determined by Wong's method,[3] were employed and the samples were read in a model 6B Coleman spectrophotometer. Later results were determined with the alkaline hematin method[3] using recrystallized hemin as a standard.[4] Previously described methods were used for the determination of calcium,[5] inorganic phosphorus,[6] cholesterol,[7] glucose,[8] "apparent" creatinine,[9] "true" creatinine,[10] alkaline phosphatase,[11] PBI,[12] and NPN.[13]

TABLE 1—Reproductive Cycle of Ewes During Various Months of the Year

Physiological state	Original ewes (born: Jan.–Feb., 1948)				1950 ewe lambs (born: May–June)			1951 ewe lambs (March–April)	
	1950	1951	1952	1953	1951	1952	1953	1952	1953
Gestation	1–6*	1–4	1–4	1–4	.....	1–4	1–4	.....	1–4
Parturition	5–6	3–4	3–4	3–4	.....	3–4	3–4	.....	3–4
Lactation and post-lactation	7–10	5–10	5–10	5–10	.....	5–10	5–10	.....	5–6
Breeding and gestation	11–12	11–12	11–12	11–12	11–12	11–12	11–12	11–12	11–12

* Months of the year.

RESULTS

Data were compiled from sampling the original ewes and their 1950 and 1951 offspring for four years. Results for the ex-

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Based on work performed under contract W-31-109-Eng-52 for the Atomic Energy Commission.

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The authors gratefully acknowledge the technical assistance of the Clinical Laboratory personnel, the Statistics Unit, and the personnel of the Experimental Animal Farm, of the General Electric Co., Richland, Wash.

amination of trends and variations were expressed as mean values and 95 per cent confidence intervals about the mean.* The over-all means for the hematological values and the significant differences obtained when mature ewes were compared with the lambs are shown (table 2).

A significant downward trend with time in the total number of leukocytes was observed in these sheep and was attributed to a decrease in lymphocyte values. Although the percentage of neutrophils apparently increased with age, there was no change in the absolute number of these cells. No changes with time were observed in monocyte, eosinophil, and basophil values.

There was a significant downward trend in erythrocyte values in the ewes over an 18-month period, but not in the hemoglobin levels. Hemoglobin values decreased appreciably during or subsequent to lambing each year.

No variation with the season was noticed in calcium, phosphorus, or alkaline phosphatase. Inorganic phosphorus was found to be lower in the original ewes than in the 1950 lambs. Alkaline phosphatase followed a downward trend with time in the adult animals, but increased in the lambs during their first year.

Nonprotein nitrogen levels were higher in adult animals but creatinine values were not. An estimation from duplicate analyses for apparent and true creatinine assembled over the period of a year showed that a factor of 0.755 ± 0.032 could be used to convert apparent creatinine values to true values, but the results from both of these analyses follow the same general pattern.

No general change with time was noticed in glucose and cholesterol in 1951 and 1952. Cholesterol increased during the spring and attained a maximum in July and August. A significant decrease in serum glucose occurred during November and December, but no comparable minimum was noticed in the lambs.

Protein-bound iodine values in the 1950 lambs declined rapidly during the period from 16 to 30 months of age.

Discussion

Higher leukocyte values were obtained with these laboratory sheep than were reported for range sheep although Holman's figure of 9.2 ± 3.1 is comparable.[14] The number of leukocytes in pregnant ewes studied by Todd *et al.*[15] was also lower than in our study. Baker [16] indicated that an age difference existed, with the highest leukocyte values occurring in yearlings while we found a decreasing trend with time over the periods observed. Individual variation probably accounted for the different levels in the seasonal patterns of the three age groups and the higher 1954 results may be due to the repetitive bleeding of only 4 sheep. The percentages of

* Complete tabular data may be obtained from the authors.

TABLE 2—Over-All Means for Hematological Values and Significant Differences Between Mature Ewes and Lambs

Constituent	No. of samples	Over-all mean	Significant differences between ewes and lambs †
Leukocytes	3,612	9.95 ± 0.25* thousand/mm.³	Original ewes < 1950 lambs; 1950 ewes < 1951 lambs; original ewes < 1951 lambs.
Lymphocytes	2,239	64.6 ± 0.7%	Original ewes < 1950 lambs.
Neutrophils	2,276	29.9 ± 0.7%	Original ewes > 1950 lambs.
Monocytes	2,044	2.5 ± 0.2%	None.
Eosinophils	2,096	2.4 ± 0.3%	None.
Basophils	2,096	0.5 ± 0.06%	None.
Erythrocytes	1,977	10.89 ± 0.15 million/mm.³	Original ewes < 1950 lambs.
Packed cell volume	296	35.0 ± 1.0%	None.
Hemoglobin	3,448	10.78 ± 0.31 Gm./100 ml.	Original ewes < 1950 lambs; 1950 ewes > 1951 lambs.
Calcium	722	12.16 ± 0.28 mg./100 ml.	None.
Inorganic phosphorus	919	5.21 ± 0.22 mg./100 ml.	Original ewes < 1950 lambs.
Alkaline phosphatase	266	1.73 ± 0.20 Bodansky units	None.
Nonprotein nitrogen	704	33.7 ± 1.0 mg./100 ml.	Original ewes > 1950 lambs.
"Apparent" creatinine	1,039	1.24 ± 0.01 mg./100 ml.	None.
Creatinine	128	0.90 ± 0.04 mg./100 ml.	None.
Glucose	631	53.5 ± 2.3 mg./100 ml.	None.
Cholesterol	611	64.6 ± 3.3 mg./100 ml.	None.
Protein-bound iodine	593	3.84 ± 0.10 μg./100 ml.	None.

* Ninety-five per cent confidence intervals about the mean. † Ninety per cent confidence level of tests.

the various classes of leukocytes, regardless of age or reproductive state of the sheep, fall into the ranges found by other workers.[15,16]

The higher erythrocyte values observed in the young sheep compared with adult ewes have been reported previously.[14,15] Holman found that the erythrocytes, hemoglobin, and packed cell volume attained minimum values in the spring months, and he speculated that, in regard to hemoglobin, this was due to inanition from suboptimal food supplies.[14] We obtained only a decrease in erythrocytes with animals fed a ration presumed to be optimum throughout the year.

As diet appears to be the main factor in maintaining the amount of inorganic phosphorus and calcium in the blood of range animals,[18-20] it is not surprising that little variation in either of these constituents was noted in this study. The wide variations in alkaline phosphatase of apparently normal adult ewes found by Allcroft and Folley [21] were also obtained here, as was a decrease in value with time. The alkaline phosphatase determinations were made only for a period of a year; therefore, it is difficult to say whether this downward trend was caused by age or pregnancy.[22]

The range of values for NPN and creatinine reported here is comparable to that found in other studies.[23,24] During 1954, urea nitrogen (BUN) instead of NPN was determined and values of 20.4 ± 0.6 in the 1950 ewes and 21.8 ± 0.9 in the 1951 ewes were obtained. The BUN analysis appeared to offer more accuracy and sensitivity than NPN.

Stickney *et al.*[25] and Zarrow *et al.*[26] found that blood glucose levels of ruminants are lower than those of carnivores and omnivores. We have obtained low results in this study and have also found this to be true of serum cholesterol. Healthy lambs of 6 months of age on a Scotland range were found to have a glucose value of 37.1 ± 9.1.[17] This figure is lower than levels reported for adult ewes,[27-29] but comparable to the data we obtained. Relatively little work has been done on the iodine fractions of ruminant serums except in dairy and beef cattle, but the PBI level of sheep serum appears to be similar to that of bovine serum.[30,31]

SUMMARY

Values for some of the hematological and blood chemical constituents of purebred Suffolk ewes and lambs are summarized. Trends and variations that occurred during a four-year study are also reported.

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Effects of Glucagon on Hypoglycemia and Ketonemia in Pregnant Ewes

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PREGNANCY DISEASE and ketosis in ruminants are characterized by hypoglycemia and ketonemia.[1,2] The occurrence of the diseases has been considered to be due to poor nutrition in the presence of unusual energy demands and to hormone insufficiency,[2,3] particularly adrenocortical hormones. Carefully purified glucagon, free of insulin, has a marked hyperglycemic effect on animals.[4,5] Since hypoglycemia is a dominant factor in this disease, the role of the hyperglycemic factor, glucagon, in the treatment of pregnancy disease requires investigation.

This paper presents the results of an experiment designed to determine the effects of glucagon on the hypoglycemia and ketonemia produced in fasted pregnant ewes.

PROCEDURE

Western, grade ewes were fed alfalfa hay *ad libitum* plus 0.25 lb. of yellow corn and 0.25 lb. of oats per ewe daily prior to fasting. The data labeled late in pregnancy were obtained from ewes fasted during the last third of the gestation period. The initial blood sample was taken after the ewes were fasted five to seven days. Twenty-four hours later, glucagon was administered intravenously. Immediately prior to glucagon administration, a blood sample was obtained. Additional samples were obtained at ten, 20, 30, 45, 90 minutes; four to five hours; and 24 hours after the administration of glucagon. This procedure was repeated on each ewe before she lambed. The glucagon was administered at the rate of 1 or 2 μg. per kilogram of body weight. Analyses of blood samples were made as previously described.[6] Blood ketones were calculated as acetone. Glucagon was dissolved in a glycine buffer solution at pH 8.5 to 9.0. Five ewes were treated as described above during their early pregnancy (3 months or less). In an attempt to investigate the interaction of glucagon and oxalacetic acid (OAA), they were administered to 4 ewes with levels of blood ketone bodies of 15 mg./100 ml. or above. Oxalacetic acid was administered intravenously at a level of 57 mg. per kilogram of body weight. Oxalacetic acid anhydride† was dissolved, immediately before injection, in a solution of 0.85 per cent sodium chloride which contained a calculated amount of 2.0 N sodium hydroxide that would give the resulting mixture a pH 6.5. This solution was given 75 minutes before the intravenous administration of glucagon. Blood samples were taken at time intervals as indicated (tables 3 and 4).

TABLE 1—The Effect of Glucagon on the Blood Glucose Levels (mg./100 ml.) of Ewes

Treatment (dosage)		Time							
		0 min.	10 min.	20 min.	45 min.	60 min.	90 min.	4 to 5 hr.	24 hr.
Early pregnancy	Av.	36(5)*	57(5)	63(4)	70(5)	.....	61(5)	47(5)	33(5)
(1 μg./kg.)	Range	17–52	24–84	41–84	59–83	.....	40–89	35–56	23–61
Late pregnancy	Av.	12(10)	26(8)	29(10)	33(10)	41(10)	.....	34(8)	15(4)
(1 μg./kg.)	Range	3–25	15–50	13–57	16–56	24–81	.....	10–50	9–23
Late pregnancy	Av.	26(7)	38(7)	45(7)	53(7)	57(6)	56(7)	37(7)	17(6)
(2 μg./kg.)	Range	14–37	19–62	29–76	29–93	41–85	31–103	17–59	11–25

* The number in the parentheses represents the number of animals.

RESULTS AND DISCUSSION

The effect of glucagon appeared to be at a maximum from 45 to 90 minutes following its administration. Only the data obtained at zero time and at 45 minutes following glucagon administration were used for statistical analyses.

The data obtained when ewes early in pregnancy were fasted and given glucagon are presented (table 1). The administration of 1 μg. of glucagon per kilogram of body weight produced a significantly (2%) higher blood sugar level.

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The authors express their appreciation to: Dr. H. W. Norton, Illinois Agricultural Experiment Station, and Mr. John Yusken, Department of Veterinary Physiology and Pharmacology, University of Illinois, for statistical analyses; and Dr. O. K. Behrens, Lilly Research Laboratories, Eli Lilly and Co., for the supply of glucagon. Purity of glucagon was approximately 50 per cent (based on the potency of crystalline glucagon), lot 208-1588-197.

† Nutritional Biochemicals Corporation, lot 6926.

TABLE 2—The Effect of Glucagon on the Level of Blood Ketones (mg./100 ml.) of Ewes Late in Pregnancy

Dosage		Time							
		0 min.	10 min.	20 min.	45 min.	60 min.	90 min.	4 to 5 hr.	24 hr.
1 μg./kg.	Av.	27(10)*	29(7)	25(9)	22(10)	22(10)	24(6)	22(8)	24(5)
	Range	12–57	11–52	10–50	4–52	8–46	9–43	6–50	13–37
2 μg./kg.	Av.	26(7)	23(7)	23(7)	22(7)	19(6)	23(7)	23(7)	32(6)
	Range	15–37	11–38	9–39	5–36	7–29	7–46	14–31	14–48

* The number in the parentheses represents the number of animals.

The effects of glucagon administered to ewes late in pregnancy at a level of 1 or 2 μg. per kilogram of body weight on the levels of blood sugar and ketone bodies are shown (tables 1 and 2). There was no significant difference between the effects of the two levels of glucagon on changes in blood sugar or ketone body levels. A significantly (1 %) higher blood sugar level and a significantly (1 %) lower blood ketone body level were observed 45 minutes following glucagon administration. These data indicate the administration of glucagon relieved the hypoglycemia and ketonemia induced in pregnant ewes by fasting. This effect persisted for at least four hours.

There was no significant correlation between the blood sugar increases and the blood ketone body decreases at 45 minutes for both levels of glucagon. In other words, a decrease in blood ketone body level could not be predicted on the basis of an increase in blood sugar level.

This laboratory has reported [7] that a derivative of OAA, administered orally, was effective in reducing the level of blood ketone bodies and elevating the level of blood sugar of fasted pregnant ewes. When OAA was administered intravenously, blood ketone bodies decreased 31 per cent and blood sugar increased 129 per cent within two hours' time.[8]

The effects of the administration of OAA (57 mg./kg.) in combination with glucagon (1 μg./kg.) on the levels of blood glucose and ketone bodies are shown (tables 3 and 4). The OAA was given 75 to 90 minutes earlier than glucagon because previous work indicated a definite response to the administration of OAA occurred approximately two hours later. Therefore, it was intended that the maximum response to both materials would coincide. The differences in the blood sugar and ketone body levels immediately prior to, and two hours after, the administration of OAA[8] were compared with data obtained in this

TABLE 3—Effect of Oxalacetic Acid (57 mg./kg.) and Glucagon (1 μg./kg.) on the Blood Glucose (mg./100 ml.) Level of Ewes Late in Pregnancy

No.	Body wt. (kg.)	24 hr. pre-treatment	OAA	Time after injection of glucagon (min.)						
				0*	15	30	60	3.25 hr.	4.0 hr.	24.0 hr.
1	39	24	23	36	29	59	84	66	53	30
2	48	30	8	69	34	80	46	27	29	25
3	51	22	16	27	32	37	....	21	34	22
4	48	23	33	69	81	....	88	65	49	16
Av.	47	25	20	50	44	66	73	45	41	23

* Time between administration of oxalacetic acid and glucagon (75 to 90 minutes).

TABLE 4—Effect of Oxalacetic Acid (57 mg/kg.) and Glucagon (1 μg./kg.) on the Level of Blood Ketone Bodies (mg./100 ml.) of Ewes Late in Pregnancy

No.	Body wt. (kg.)	24 hr. pre-treatment	OAA	Time after injection of glucagon (min.)						
				0*	15	30	60	3.25 hr.	4.0 hr.	24.0 hr.
1	39	11	15	13	13	7	13	....	4	12
2	48	21	35	21	22	22	25	30	....	41
3	51	24	26	19	22	34	....	19	15	19
4	48	17	22	22	22	....	22	27	22	32
Av.	47	18	25	19	20	21	20	25	14	23

* Time between administration of oxalacetic acid and glucagon (75 to 90 minutes).

experiment. In this experiment, data obtained at zero time and one and three fourths hours after the administration of OAA and 15 minutes after the administration of glucagon were used for statistical analysis. Analysis of the data obtained at these specific times indicated there was no significant difference between the effect of OAA[5] and the effect of OAA and glucagon on blood sugar and ketone body levels.

SUMMARY

The intravenous administration of glucagon appeared to have therapeutic value in the treatment of hypoglycemia and ketonemia induced by fasting. Intravenous administration of glucagon at a level of 1 or 2 μg. per kilogram of body weight to pregnant ewes fasted five to seven days produced a significant rise in blood glucose and a significant decrease in the level of blood ketone bodies. The maximum antiketogenic action was attained 45 and 60 minutes following glucagon administration for ewes early and those late in pregnancy, respectively. Glucagon and oxalacetic acid did not have an additive effect on changes in blood sugar and ketone body levels.

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The Natural Protection of Sheep from External *Beta* Radiation

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WHILE differences in the susceptibility of many animal species to ionizing radiations are well known,[1-5] the radiosensitivity of some has never been investigated. Until recently, there was little interest in whether sheep reacted differently to ionizing radiation. While there was no apparent reason to expect sheep to differ at all from other mammals in their susceptibility, little thought beyond this point had been given to the question. It was, therefore, quite unexpected to hear in April and May of 1953 that sheepmen, wintering sheep north of the Nevada Proving Grounds, attributed heavy losses in their flocks to residual radiation from the atomic tests of that spring. When the reports of the many radiological safety teams who monitored these areas were analyzed, it was difficult to understand how these claims could be valid, since the highest integrated *gamma* radiation dose found in those areas was only 5 r and the integrated *beta* radiation dose could not have exceeded 10 to 15 rep.

Previous animal experimentation [6-8] indicated that either these dosage measurements were in error by a factor of 50 to 100 or that sheep were many times more susceptible to radiation damage than any mammalian species about which such information was known. Otherwise, the high death rate among the sheep in southern Utah and northern Nevada that spring was more likely attributable to the existing severe drought, poor forage and the remarkable recent increase in noxious plants, and to the resulting poor condition of the pregnant ewes before the long spring drives.

When investigating teams of various installations of the Atomic Energy Commission arrived in Utah, the great majority of the sick sheep had either recovered or had died and been burned or buried. Some of the buried sheep were examined successfully several months after death by Dr. W. J. Hadlow, Hamilton, Mont., and members of this laboratory. Local veterinarians, however, were unable to determine the cause of the malady and had not observed such a prostrating disease before. The disease was manifested by progressive stages of erythema, desquamation, and papule and pustule formation on the face, ears, sides, and back. In lambs, masses of tissue protruded from the cheeks and lips. The loss of hair about the muzzle and the tips of the ears with ulceration and crust formation was suggestive to some observers of radiodermatitis. These skin lesions caused others to hazard the guess that the whole disease was due to exposure to ionizing radiation. It is well known, however, that in most [2-5] other animals about 500 rep are required to cause epilation and more than 2,000 rep are required to produce ulceration with scab formation. Often 5,000 rep are needed to produce ulceration. The animals which were found extant months later and which were said to demonstrate the typical injury had persistent skin lesions located in a hit-or-miss fashion on their flanks and the sides of the head, but rarely on the back (fig. 1). These lesions appeared traumatic and were heavily soiled because the wounds had allowed dirt to reach the base of the wool and diffuse widely (fig. 2 and 3).

Studying these wounds, one could see how they resembled the eschar of radiation damage, as well as other dermatitides, but it was also evident that the preshearing thickness of wool must be an excellent protector against *beta* irradiation. Dirt particles falling on the surface of the wool

This work was done under the auspices of the U.S. Atomic Energy Commission. However, the opinions and conclusions presented in this paper are not necessarily those of the Commission. This paper constitutes the final report of this work, which was submitted as a preliminary report for the information of the Division of Biology and Medicine of the Commission in March of 1954.

The authors acknowledge with appreciation the technical assistance of Dorothy B. Hale and Julie M. Wellnitz; the veterinary consultation of Robert Thompsett, D.V.M., Los Alamos; Col. John Rust, V.C., and Lt. Col. Bernard F. Trum, V.C., Oak Ridge, Tenn.; W. J. Hadlow, D.V.M., Hamilton, Mont.; and Charles L. Davis, D.V.M., Denver, Colo.; and the aid of Thomas N. White, Ph.D. (deceased), in determining theoretically the extent of shielding afforded by raw wool.

fail to reach the skin (*see* fig. 2) because of the compactness of the fleece and also because of the adhesive qualities of the sebum coating, which continuously moves up the fibers away from the skin surface. Horses, which have relatively little hair compared to sheep, have been injured by radioactive fall-out from atomic tests (fig. 4), but these injuries are characteristically along the backbone and on top of the head. The lesions on the horses were circular, multiple, and variable in size, distribution, and depth, while those on the sheep were linear, isolated, and uniform.

Radioactive fall-out settles slowly from the upper atmosphere. It consists chiefly of small particles of substances emitting *beta* radiation. Since *beta* radiation is unable to penetrate matter deeply, it would seem that sheep (whose thick fleece prevents dust particles from coming in contact with the skin) would have excellent skin protection from *beta* radiation and, therefore, from atomic fall-out unless, for some unknown reason, their skin was unusually delicate and very susceptible to irradiation damage. The following experiments, which were done to determine how susceptible the skin of the sheep is to *beta* irradiation, appear to resolve the controversy.

EXPERIMENTAL PROCEDURES

The *beta* radiation used in these experiments was delivered from Sr^{90}—Y^{90} sources contained in special source holders with thin, stainless steel apertures. One source, manufactured by the Oak Ridge National Laboratory, had a round aperture of 15-mm. diameter and a *beta* radiation dose rate of 5,400 rep per minute at the surface. The other applicator, manufactured by the Tracerlab Co., had a 5-mm. diameter aperture and a delivery rate of 1,944 rep per minute. These dose rates were determined by the extrapolation chamber method. Exposure of the skin or wool surfaces to *beta* radiation was accomplished by placing the applicator lightly but firmly against the surface

Fig. 1—Entire left flank of a sheep obtained in Cedar City, Utah, and said to have been injured six months previously, during atomic weapons tests. Two diagonal cleftlike breaks in the wool can be seen.

Fig. 2—Same area pictured in figure 1, now sheared, showing the appearance of the scarred skin and the manner in which the local loss of wool production through disease allowed dirt to reach the skin and discolor adjacent areas of normal wool. The undamaged wool, as seen around the edge of the shaved patch, clearly prevented particulate debris from reaching the skin surface.

for a sufficient period to deliver the desirable total dose to the area. Figure 5 shows the two applicators in use. The operator is protected from exposure by the long rod to which the applicator is attached and by a lucite shield.

The sheep used in this experiment were of the fine-wool type, which is characteristic of the western range. Two experiments were done. The first was designed to measure the sensitivity of the unprotected skin of the muzzle, ears, and flanks to *beta* radiation applied directly to it. The second experiment was done to determine the protective effect of the normal fleece against *beta* radiation. In the first experiment, 2 sheep were used. One side of each sheep was sheared with electric clippers as closely as possible. On the shaved area, 18 blocks, 3 inches square, were marked off so that there were three rows with six blocks each running from the shoulder to thigh. The large applicator was placed directly on the skin in the center of each block (fig. 5). Each block received only one exposure. The six exposures in each row were: 2,500, 5,000, 10,000, 15,000, 20,000, and 25,000 rep. The order of the doses was reversed in the second row and again in the third to determine whether sensitivity to radiation varied according to location. The muzzle, corners of the mouth, and ears of each sheep were also shaved and each given exposures of similar magnitude with the small applicator. Doses of 10,000 rep and below were applied to the areas on the right side of the head and doses of 15,000 rep and above were delivered to the left side of the head. The exposed areas were observed daily for 100 days and photographic records were made of the progress of the lesions at weekly intervals or at times when significant changes had developed.

In the second experiment, the same 2 sheep were used again. Four exposure areas were marked off along the unsheared flank which corresponded to those in the middle row on the opposite sheared side. Moving caudally, *beta* radiation doses were delivered to a spot in the middle of each exposure area by placing the large applicator lightly against the surface of the wool. The respective doses were: 5,400, 16,200, 48,600, and 145,800 rep. The staple length of the wool averaged 33 mm. The four exposure areas on 1 sheep were sheared immediately after exposure so that changes in the skin could be observed. The exposure areas on the second sheep were left unsheared in order to observe the occurrence of epilation.

The unshaved ears and the corners of the mouth of a third local ewe (in spring wool) were exposed to 5,000 rep on the right side and to 10,000 rep on the left. The staple in these areas was between 5 and 10 mm. In both experiments, skin biopsies were obtained at various intervals from different lesions for histological study.

Experimental Results

Daily gross observations of the exposed sheared areas revealed that 25,000 rep of *beta* radiation caused ulcerative lesions which were manifest in eight days. One ewe developed a superficial ulceration in 13 days in the area that received 2,500 rep, but the skin of the second ewe was unaffected by this dose. Ulcerations appeared in both sheep in the other exposed areas between the eighth and the thirteenth days. Below 10,000 rep, the loss of tissue was confined to the epidermis, so that scabbing was minimal. The ulceration after 2,500 rep in the 1 sheep was so superficial that it amounted to little more than epilation. Above 10,000 rep, the ulcerative process appeared to extend into the dermis progressively deeper as the dose was increased, so that at 25,000 rep the ulceration ultimately extended deep into the skin.

Figure 6 shows how the lesions appeared grossly three weeks after exposure. Weeping and scabbing are seen to a slight extent in the lesions caused by 15,000, 20,000, and 25,000 rep. Below these dosages, only the appearance of epilation can be

Fig. 3—The opposite side of the same ewe shown in figures 1 and 2.

seen and the three squares exposed to 2,500 rep fail to show any effect. Figure 7 shows that at nine weeks the epilated areas at the lower doses are still devoid of wool, while heavy dark (hemorrhagic) scabs are present in the centers of some of the areas exposed to the three higher doses. These lesions were typical of ulcers of radiodermatitis or radiation burns. They developed slowly by first epilating and weeping and then sloughing the superficial layers and forming crusts over ulcerations that extended deeper with time and were protracted in healing.

Wool growth stopped in all areas of exposure, except in the area of lowest dose (2,500 rep) in the more resistant ewe. The areas of suppressed wool growth were in most cases larger than the aperture of the applicator and seemed to increase in area as the dose increased (table 1; fig. 11). The epilated areas reached their maximum size about the third week after exposure. At eight to nine weeks after exposure, the lesions produced by 10,000 rep or less were no longer weeping and were healed and re-epithelialized. New wool growth did not occur in the centers of the lesions caused by greater than 5,000 rep but occurred to a limited extent at the periphery of these areas, so that when the last observations were made, 32 weeks after exposure, the epilated areas were slightly smaller than maximally.

Lesions on the ears and muzzle of both shaved sheep were similar in development and appearance to those on the sides, except that the appearance of the lesions on the ears was more delayed than in other areas and scabbing and ulceration was neither as extensive nor as prolonged (fig. 8).

Fig. 4—Three horses with beta radiation injury from atomic bomb fall-out. The gross characteristics of the lesions seen here are the speckled distribution along the backs rather than the flanks, the numerous round, irregular-sized areas of depigmentation and superficial ulceration, and the increase in the area of injury through overlapping of adjacent lesions.

These horses were within 2 miles of an atomic explosion in a series of tests that preceded the one to which the sheep were said to have been exposed.

The unsheared areas on the head of the third ewe which was exposed to 5,000 rep (the ears and corners of the mouth) on the right side and 10,000 rep on the left side did not develop lesions. The only observable effect of the exposures was partial epilation about the mouth on the side exposed to 10,000 rep. This epilation occurred about four weeks after radiation and still persisted at the last observation 32 weeks after the exposure.

The exposures of the unsheared wool of the sides produced interesting results. Ten days after exposure to 16,200 rep or more, the wool under the exposed areas could be plucked without force (fig. 9). The lowest dose, 5,400 rep, did not produce visible damage to the skin or follicle and wool did not epilate from this area. Wool plucked from irradiated areas lacked the characteristic bulb of wool plucked from normal skin (fig. 10) and had the characteristic appearance of hair from damaged follicles.[4] Ulcers developed between the second and third week after exposure to 145,800 rep in both sheep and with 48,600 rep in 1. When the last observation was made, 44 weeks after exposure, only the ulcer caused by 145,800 rep had a thick scab, while the area exposed to 48,600 rep had an atrophic scar. The other areas were not demonstrable at this time. New wool growth obscured the areas exposed to 16,200 rep by the four-

TABLE 1—The Size of the Epilated Area on Sheared and Wool-Bearing Skin of a Sheep Exposed to Various Amounts of Beta Radiation from a Source of Constant Area

Dose (rep)	Area of epilation*	
	Sheared skin (mm.²)	Wool-bearing skin (mm.²)
2,500	225*	.....
5,000	400	.....
5,400	.....	0
10,000	475	.....
15,000	594	.....
16,200	.....	1,330
20,000	700	.....
25,000	700	.....
48,600	.....	2,500
145,800	.....	1,935

* Actual area of aperture in radiation applicator.

teenth week. Wool growth did not resume after the higher exposures. The areas of epilation were larger than the diameter of the aperture because of the greater area subtended by the solid angle from the source (table 1; fig. 11). By 32 weeks, these epilated areas were reduced to about one half of their maximum size.

Histological Observations

The skin lesions and organs of 6 sheep obtained from the diseased flocks in Nevada and Utah and the skin lesions of 1 horse, which was known to be injured by *beta* radiation from fall-out, formed the baseline for the histological investigation. Dr. Hadlow performed necropsies on 3

Fig. 5—Ewe being exposed experimentally to beta radiation from two applicators containing Sr^{90}–Y^{90}. One applicator is being held against the shaved skin of the flank, which has been marked off in squares that will receive different radiation doses. The second applicator is being used to expose the shaved skin of the corners of the mouth.

sheep and Col. John Rust and Lt. Col. B. F. Trum, Oak Ridge, Tenn., on 2 sheep and the horse. All of the sections of various naturally occurring skin lesions available to the Office of the Regional Pathologist, U.S. Department of Agriculture, Denver, Colo., were loaned to the Laboratory for study by Dr. Charles L. Davis. An additional sheep was obtained alive from Cedar City, Utah, in the fall of 1953 in order that the lesions could be studied grossly and microscopically and so that the sensitivity of the sheep from that part of the country could be compared to those obtained for experiment from the Rio Grande Valley in New Mexico.

Biopsy specimens were obtained from the experimental lesions caused by 5,000 15,000, and 25,000 rep in 1 sheep six weeks after exposure and from a lesion caused by 25,000 rep nine weeks after exposure in the same sheep. Fifteen months after the beginning of the experiment, sections were obtained from the other sheep representative of lesions caused by 5,000, 10,000, 15,000, 20,000, and 25,000 rep to the sheared skin, and by 16,200, 45,600, and 145,800 rep to the surface of the wool over the skin on the other side. Five representative sections were obtained from the lesions of unknown cause on the sides of a sheep from Cedar City, Utah, approximately 15 months after the spring bomb tests in Nevada.

The Lesion from the Utah Sheep.—This lesion consisted grossly of a stiff, thickened, epilated hide covered in the central areas by thick irregular crusts that were firmly adherent to the dermis.

Microscopically, two stages of this lesion were seen. The first stage was characterized by epidermal abscesses; epidermal hyperplasia with great increase in size of the epidermal rete pegs; persistence of sebaceous glands, which in one instance were also hyperplastic; rapid disappearance of the sweat glands; follicular atrophy and disappearance; and the formation of kera-

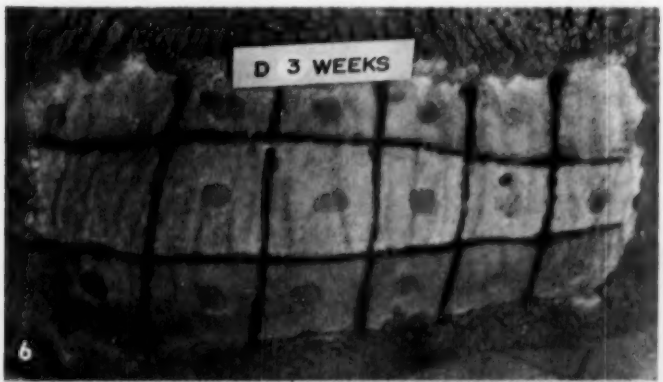

Fig. 6—Sheared skin of sheep three weeks after the exposures. The doses were 25,000, 20,000 15,000, 5,000, and 2,500 rep in the top and bottom rows from left to right, and reversed in the middle row.

No lesion is seen in the areas exposed to 2,500 rep. The epilation becomes increasingly prominent from the lowest to the higher doses where superficial ulceration can be seen.

Fig. 7—Same skin areas (fig. 6) nine weeks after exposure showing persisting epilation in healed areas exposed to low dosages and dark hemorrhagic scabs still present in the areas exposed to 15,000 rep or more.

tinous cysts in follicular shafts and sweat gland ducts (fig. 12 and 13).

The second stage was atrophic, as far as the epithelial changes were concerned, but was hyperplastic in regard to the connective tissue elements (fig. 12 b and 13 a, b, and c). The atrophy of the epidermal appendages was not complete, in that scattered throughout the sections there were occasional wool follicles, sweat glands, or sebaceous glands. Sweat glands were more uniformly absent than the other two elements and persisting follicles were the commonest remnants. Those arrectores pilorum muscles that were found were extremely atrophic and, in those sections where atrophy was extensive and sclerosis extreme, no such muscular remnants could be found at all. The papillary layer and the reticular layer were combined into a greatly thickened dermis composed of densely packed hypertrophied eosinophilic collagenous fibers and bundles. This sclerosis seemed to be most prominent in the deepest portion of the reticular layer, where the sweat glands are normally located. There was no evidence of regenerative attempts in this severely scarred lesion.

The Lesion Produced Experimentally by Beta Radiation.—The numerous facets of the typical lesion of *beta* ray dermatitis

Fig. 8—Six areas, defined by ink, on the angle of the mouth and the tip of the ear exposed to 15,000, 20,000, and 25,000 rep of beta radiation. Epilation without deep ulceration has occurred but is not prominent.

are shown in figure 14, which shows panoramically the histological features of a section of an entire radiation ulcer. As one examines such a lesion, moving from the normal surrounding skin inward to the most severely damaged portion, the following epithelial changes are seen which characterize the subacute phase of such a "burn": (1) atrophic wool follicles persisting in the presence of sebaceous and sweat glands of normal appearance; (2) hyperplastic wool follicles in the absence of sebaceous and sweat glands; (3) mark-

Fig. 9—Four areas of wool of a ewe exposed, from right to left, to 145,800, 48,600, 16,200, and 5,400 rep, respectively. The site of the latter exposure is marked by a large inked mark while the others are seen as large areas from which the wool was plucked easily.

edly hyperplastic, acanthotic epidermis, wool follicles with no sign of other appendages; (4) loss of epidermis through dyskeratosis but persistence of the deep follicular hyperplasia; (5) eschar formation by exudation of proteinoid fluids, leukocytes, and sloughing epithelial debris; (6) loss of the dermal papillary layer while the arrectores pilorum are somehow preserved; and (7) moderate leukocytic exudation into the connective tissue without a fibroblastic or a collagenous proliferation.

The healed center of the ulcer finally looks like figure 15 a, which shows a thin skin devoid of follicles, glands, or dense scarring, but the persistence of the arrectores pilorum (fig. 15 d). Epidermal hyperplasia may persist for a long time over the area of former ulceration, but this is not accompanied by hyperplasia in the epidermal appendages.

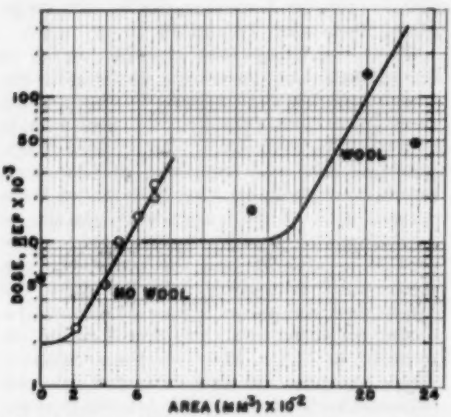

Fig. 11—The effect of increasing doses of beta radiation from a 15-mm. diameter applicator on the area (mm.²) of the resulting lesions, when the skin is sheared and when it is covered with fleece having a staple length of 33 mm. The latter curve is discontinuous and its exact form is dubious.

This figure shows that as the dose to an area of shaved skin is increased, the amount of lateral scattering to adjacent skin increases proportionally and lesions of progressively larger areas result. It also shows that wool covering the skin serves at first to protect the skin by decreasing the exposure (chiefly through the principle of the inverse square law). When the total dose becomes large enough to produce skin lesions, the areas are larger than if the source was against the skin because of solid angle considerations.

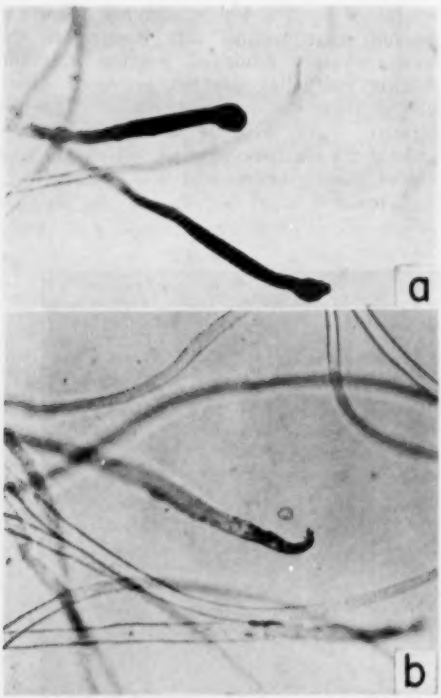

Fig. 10—Photomicrographs of the ends of wool fibers stained with acid fuchsin showing: (a) the bulbous portion of wool fibers from normal skin and (b) the loss of the bulb in fibers plucked from irradiated skin.

This phenomenon is nonspecific and occurs with injuries, other than irradiation, which result in epilation.

Pertinent Histological Features of Some Naturally Occurring Sheep Skin Diseases.—The following skin diseases were observed histologically in this study: contagious ecthyma, actinobacillosis, bluetongue, sore muzzle, filarial dermatosis, infectious pustular dermatitis, ulcerative bacterial stomatitis, papillomatosis, and viral granulomatous dermatitis. While these diseases differ from one another in many ways, they all have some features in common to the others, which appear to result from the structural and functional peculiarities of sheep skin. These mutual features appear to be: epilation, crusting exudation, intraepithelial abscess formation, epidermal hyperplasia, keratinous cyst formation, early sweat gland destruction or atrophy, and fairly uniform susceptibility of the different dermal appendages to inflammatory trauma. These pathological manifestations are common to most inflammatory diseases of the skin, because of the

basic general principles which are involved in the morphogenesis of the dermatitides in sheep. Their elucidation appears to be pertinent to an understanding of the differences between the "unknown" Utah skin lesion and that produced experimentally by *beta* radiation.

One of the most obvious principles is that the large sweat glands and their patent ducts act as a direct portal for infection to reach rapidly the depths of the corium. Irregularities in these ducts and their partial obstruction by keratinous debris in the epidermis lead to localized foci of infection and resulting abscess formation in these areas, giving rise to intraepithelial, superficial, and deep dermal infections singly or simultaneously. Wool growth is a sensitive measure of the well being of the animal and especially of the local area of skin, so that with most infections, of all intensities, epilation is a com-

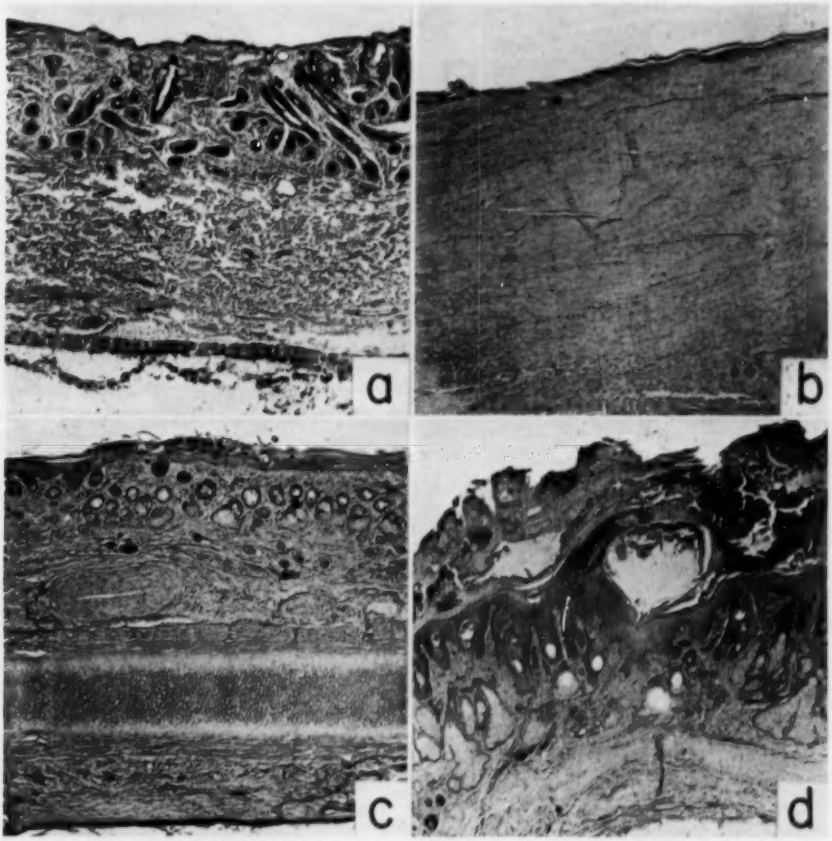

Fig. 12—Photomicrographs of histological sections of skin lesions from 2 Utah range sheep.

a) A dry poorly wooled area showing, on the left, atrophy of wool follicles, isolated normal sebaceous glands, and the almost complete absence of sweat glands. x 48.

b) A completely epilated sclerotic scar, showing the complete loss of all dermal appendages and remarkably dense hypertrophic sclerosis of both the compact and loose areolar strata of the dermis. x 48.

c) A scaly epilated area from the ear, showing keratotic cysts in sweat gland ducts, atrophy of follicles, and persistence of sebaceous glands. x 56.

d) Heavily crusted epilated area from the ear showing an intraepithelial abscess, persistent keratotic cysts in sweat gland ducts, complete atrophy of follicles with coexistent intense epidermal hyperplasia, and hypertrophy of sebaceous glands. x 48.

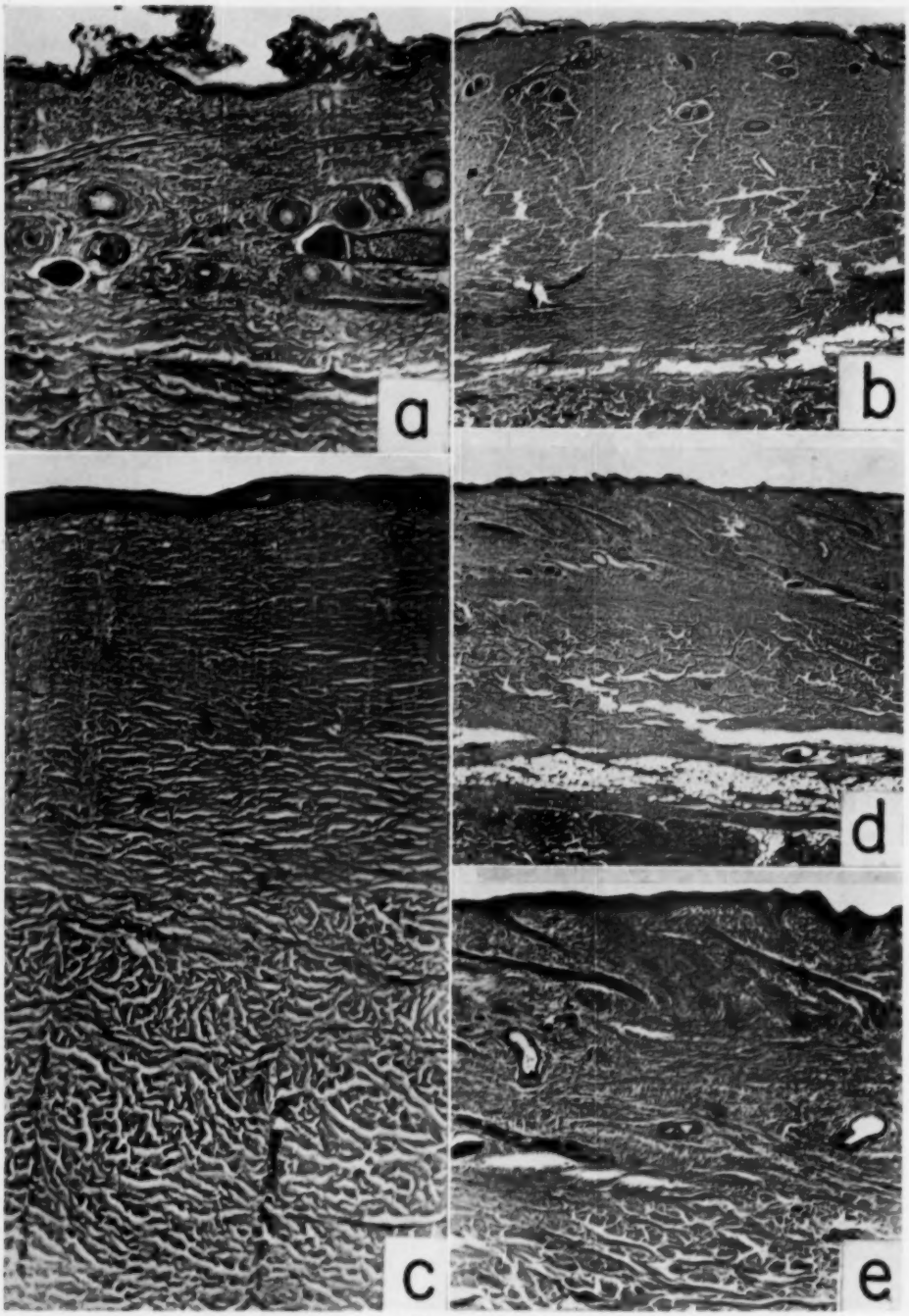

Figure 13

TABLE 2—Comparison of the General Histological Features of Infectious and Radiation-Induced Skin Lesions in Sheep

Structure	Infection		Radiation	
	Strong	Weak	Strong	Weak
Epidermis	Hyperplasia, keratinous cysts	None	Dyskeratosis and atrophy	Hyperplasia
Dermis				
a) Sebaceous glands	Atrophy(3)*	Hyperplasia	Atrophy(3)*	Atrophy
b) Follicles	Atrophy(2)	Epilation	Atrophy(1)	Epilation, hyperplasia
c) Sweat glands	Destructive atrophy(1)	Keratinous cysts, abscesses, atrophy	Atrophy(2)	No effect
d) Connective tissue	Intense fibroplasia with dense scarring	Reactive stimulation with fibroplasia	Progressive collagenous degeneration and ulceration	Lack of noticeable reaction or scarring
e) Arrectores pilorum muscle	Complete loss	No effect to atrophy	No effect	No effect

* (1), (2), (3) = relative order of susceptibility and atrophy.

mon feature occurring as the result of temporary or permanent suppression of follicular mitotic activity or atrophy and destruction of the follicle.

There appears to be some difference in the susceptibility of the various dermal structures to infectious trauma, but the difference is not great enough to lead to protection of one structure while the other is completely and selectively lost. Sweat glands and wool follicles are most sensitive and about equally so. Sebaceous glands appear to be the least sensitive, so that they are often spared while the other structures are lost. The small differences in susceptibility, however, are often overcome by the uneven distribution of the traumatic infectious agent throughout the skin, so that the dermal appendages in these diseases often appear to be destroyed in the hit-or-miss fashion resulting in a disorderly microscopic appearance. Because of the deep penetration of these diseases, there is severe deep as well as superficial scarring, which is marked histologically by dense collagen production and dermal sclerosis of keloid quality. The arrectores pilorum of the skin are similarly susceptible to inflammatory destruction by bacterial action, so that they fail to persist in the resulting scar.

Discussion and Conclusions

The features of an infectious skin lesion of sheep are contrasted in table 2 with those of the experimentally produced *beta* ray dermatitis with regard to the effectiveness of relative strengths of the etiological agent. It can be seen from this comparison that there are many features of each which resemble those of the other, which would cause them to be confused on gross or superficial microscopic examination. The

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Fig. 13—Comparison of the microscopic appearance of the epilated scarred lesion of a Utah range sheep with that of completely epilated scarred areas in the same sheep produced experimentally with 5,000 rep of beta radiation. Photomicrographs a, b, and c are of the naturally occurring lesions and d and e are from the lesion made by irradiation of another area. Photomicrographs a, c, and e are magnified x 81, and b and d are magnified x 53.

Remnants of hair follicles and sebaceous glands persist in areas of the sclerotic dermis. Two atrophic remnants of arrectores pilorum muscles are to be seen above the follicular remnants. Sweat glands are completely gone.

b) Another such area under lower magnification, in order to demonstrate how the skin has been thickened about threefold by a sclerosing process in the deeper portion of the reticular layer of the dermis.

c and e) Section (c) from the center of the lesion of which photographs a and b are the margins. It shows the complete loss of dermal structures and the extreme thickening of the dermis by means of a dense deposition of collagen. When compared with e, photograph c shows the reduced thickness of the skin in an irradiated area. The reticular areas in the deep layer of the dermis in each (c and e) are matched side by side for this purpose.

d) An experimental irradiated lesion at the same magnification as b, showing how thin the irradiated skin is in comparison, the persistence of arrectores pilorum, and an occasional sweat gland duct. These muscles and ducts are seen better in photograph e, which compared to a, indicates that the muscles in the irradiated skin are not atrophic as they were in the natural lesion.

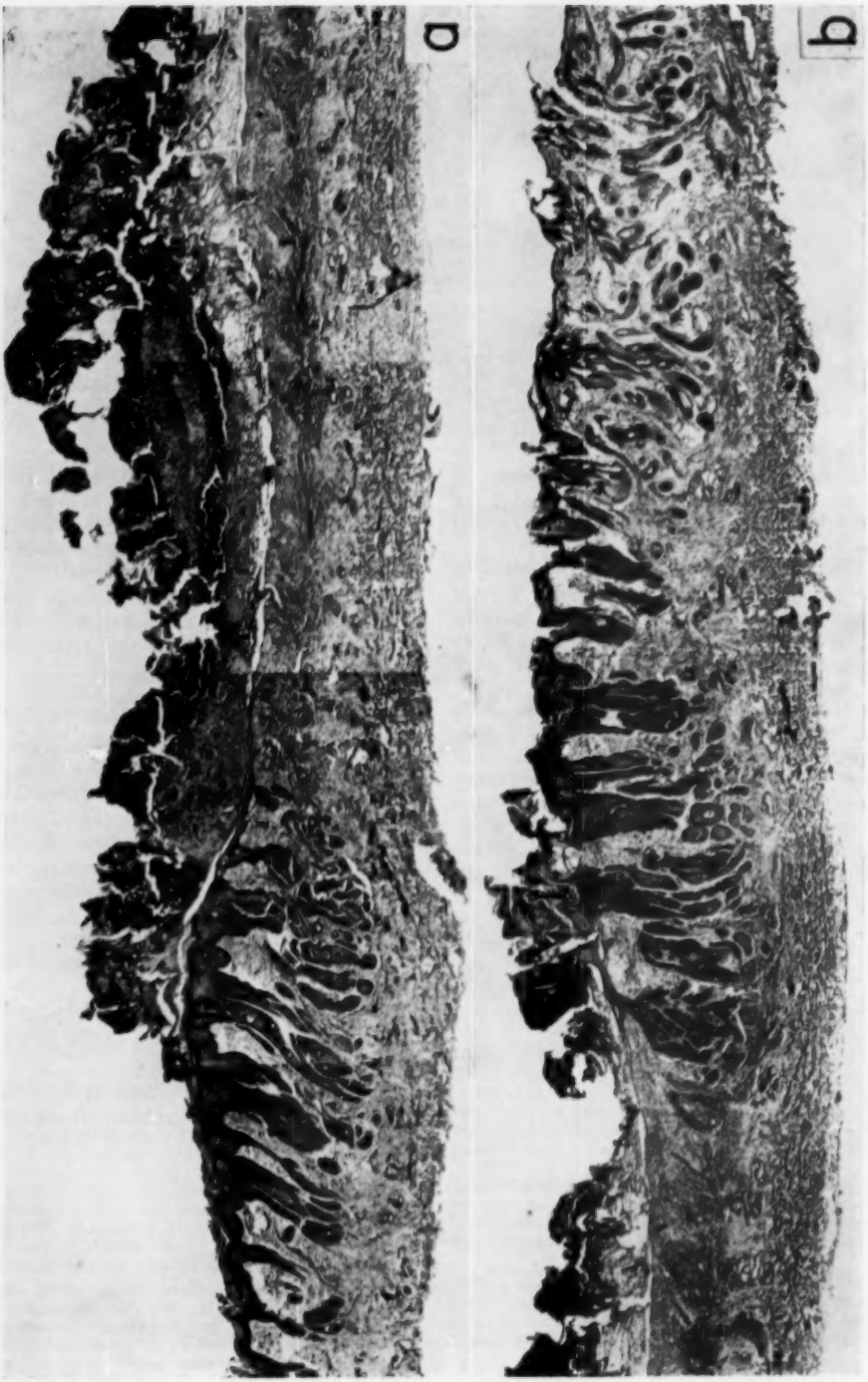

Figure 14

comparison also shows, however, that there are many features which are so dissimilar that their microscopic differentiation would seem not only feasible but reasonably facile and accurate.[1, 2, 5, 6]

This study shows that the skin disease of the Utah sheep was not the result of exposure to ionizing radiation. It shows that the lesions found in the range sheep failed to fulfill the histological requirements for them to have been caused by *beta* irradiation, but they did have all of the aspects and qualifications of an infectious process. Study of the naturally occurring sheep disease called infectious pustular dermatitis showed that the Utah disease was an apparently fulminating variant of this disease and that there were no incompatibilities with a positive diagnosis of this disease in the cases studied. On the other hand, the progressive atrophy of the arrectores pilorum in these lesions and their complete loss and disappearance in a hyperplastic sclerotic dermal process completely deny the possibility of these lesions being the result of radiation damage. The test of this concept is demonstrated by the study of the skin lesions of the range horses from Nevada that were known to have been contaminated by radioactive fall-out. The histological features of radiodermatitis in the horse are demonstrated in figure 16. Figure 16 c shows the telltale persistence of the arrectores pilorum in the scar under a radiation ulceration in an area of skin where all other dermal structures have been completely wiped out. Such preservation of these minute muscular structures was seen repeatedly in the lesion produced in sheep experimentally with *beta* radiation but never in any of the examples of the natural dermal disease of the range sheep.

Indirect evidence that the skin disease of the range sheep was not caused by ionizing radiation was obtained from the study of the threshold dosage requirements for radiological epilation, crusting, and ulceration. The failure of 1 of the 2 sheep to develop any lesion at all after exposure to 2,500 rep of the sheared skin in three different areas indicates that the threshold for epilation and retardation of wool growth lies between 2,500 and 5,000 rep. Since the highest integrated dose in the field in the most heavily contaminated areas that were found was but 5 r of *gamma* radiation and since the *beta-gamma* ratio at most could be 10:1 and probably was not over 2:1, there is a wide discrepancy between the exposure that was possible and that which would have been required to produce minimal damage to the sheared skin of sheep.

The study also showed that the sheared skin of the face and ears was more resistant to the effects of radiation than the skin of the flanks. Furthermore, it indicated that the unsheared skin of the face was not epilated by 5,000 rep of *beta* radia-

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Fig. 14—Panoramic photomicrographs (x 53) of an entire skin section made from an area of sheared sheep skin exposed 33 days before biopsy to 25,000 rep of beta radiation. The right end of photograph a should be joined with the left end of b.

At the far right of the section (b), the skin is normal as shown by the thin epidermis, the irregularly curving thin hair follicles containing darkly staining wool fibers and bulbs, numerous sebaceous glands associated with the follicles in the outermost strata of the dermis, and the dilated sweat glands of the loose areolar connective tissue of the deepest layer of the dermis.

About halfway from this perfectly normal skin to the radiation ulcer in left center there is an area of atrophy of some of the dermal structures: sebaceous glands and sweat glands persist, but the wool fibers are missing and the follicles are atrophic and inconspicuous. More to the left, the sebaceous glands are gone, while atrophic remnants of the deep sweat glands can still be seen. Also in this area, the surviving squamous epithelium of the epidermis and wool follicles is hypertrophic, due to reparative hyperplasia.

As the margin of the crater is approached more closely, this hyperplasia is seen to be so extreme that the thickness of the skin is increased. The group of follicles at the margin of the ulcer are less hyperplastic, while the superficial epithelium over these follicles has failed to undergo any hyperplasia at all.

The ulcer crater, which begins to the left in b and extends to the far left in a, is covered by a thick, dark-staining amorphous scab in which cornified epithelial and other cellular debris can be seen along with remnants of wool fibers and follicles.

The right-hand half of the scab contains loculated areas of fluid in the exudate. The ulcer appears to destroy about half of the compact layer of the dermis, without loss of the areolar layer. Between these layers, remnants of sweat glands can be seen. Just below the line of cleavage between the scab and dermis, dark, slanting, thin structures can be seen which are persisting arrectores pilorum.

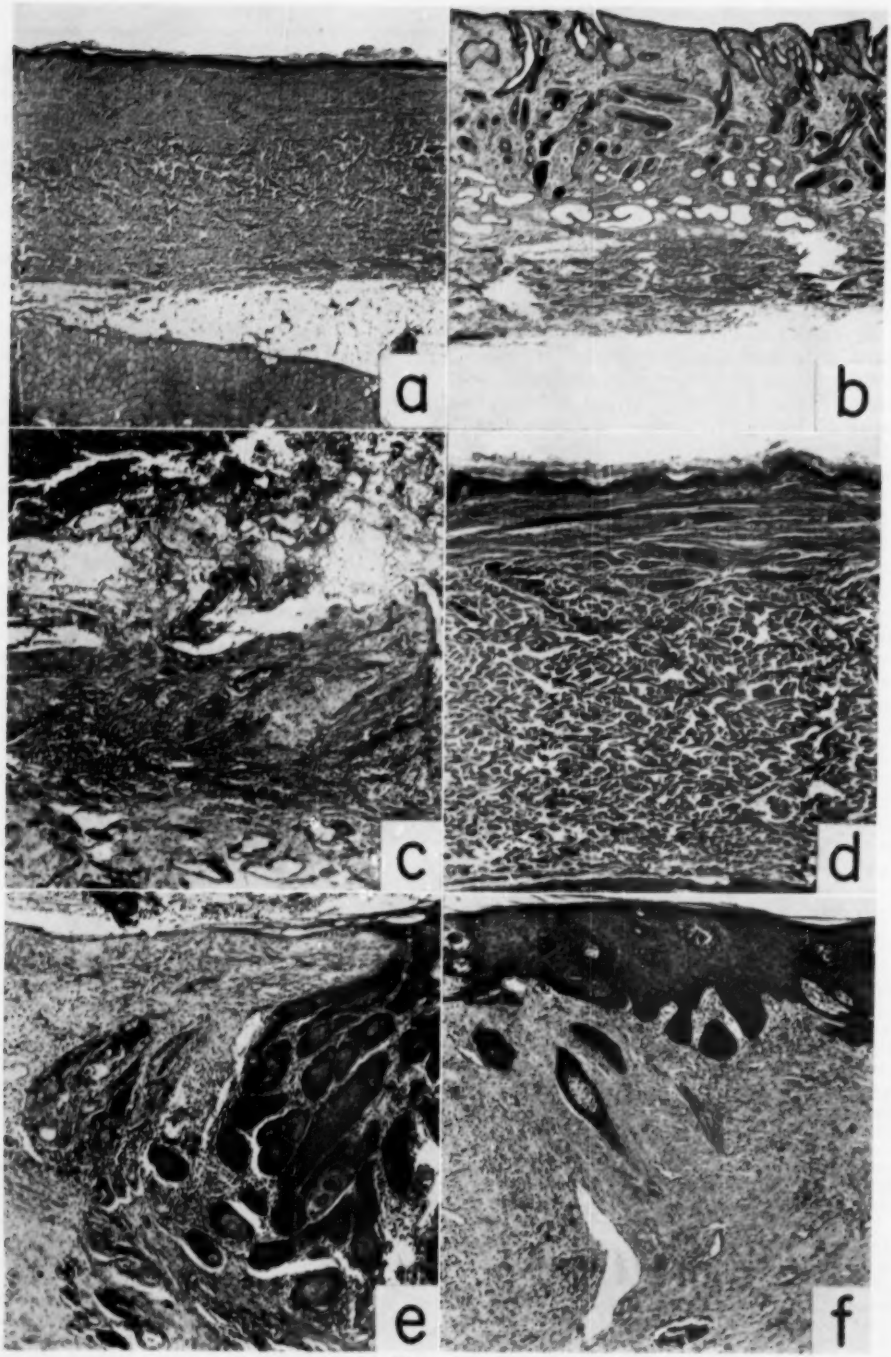

Figure 15

tion but was epilated by 10,000 rep, which failed, however, to cause ulceration. The experiment in which the full wool was exposed to *beta* radiation supported further the concept of protection being afforded by the wool. In that experiment with wool 33 mm. long, having a mass 43 mg. per centimeter^2, 16,200 rep was required at the surface before epilation without ulceration was produced. A scar resembling microscopically those seen in the Utah range sheep was not obtained in the laboratory until over 145,000 rep was applied to the surface of the wool. Infinite dosages of *beta*-emitting substances of this magnitude have never been encountered in the field, except in the immediate region of ground zero where destruction by blast and heat is maximum. If the lesions on the flanks of the sheep obtained from Cedar City, Utah, had been caused by radiation, it is estimated that an exposure of 50,000 to 150,009 rep would have been required to produce them.

These experimental observations of the large amount of protection afforded the skin by the covering of wool were confirmed by the following theoretical considerations of Thomas N. White of this Laboratory.[9] Attenuation of the *beta* dose rate by wool was calculated by the formulas of Rossi and Ellis,[10] using the following assumptions: length of staple, 3.3 cm.; surface density of wool, 43 mg. per centimeter^2; and diameter of the applicator, 5/8 inch. The source was considered as a thin truncated cone with the skin where the apex of the cone would be, and the dose rate ratio was assumed not to fall off too rapidly as one moves away from the center line. Two different *beta* ray attenuation coefficients for wool (2.3 cm.^{2}/Gm., extrapolated from Rossi and Ellis' data, and 7.8 cm.^{2}/Gm. from the empirical expression of Goodman [11]) were used. Calculations were also made for infinitely thin and infinitely thick sources with respect to self-absorption in the active material.

The formulas derived for the ratio of the dose rate on the skin to the dose rate at the surface of the applicator were:

Thin source:

$$\frac{E_1(b+c) - E_1[(b+c)\sec\theta]}{E_1(b)} \approx \frac{a(b+c)E_2(b+c)}{E_1(b)}$$

Thick source:

$$\frac{E_2(b+c) - \frac{E_2[(b+c)\sec\theta]}{\sec\theta}}{E_2(b)} \approx \frac{a[E_2(b+c)+(b+c)E_1(b+c)]}{E_2(b)}$$

Where: b is thickness of filter in mean free paths; c is thickness of wool in mean free paths; θ is half the angle subtended by the source at the skin; a is sec $\theta - 1$; and the E functions are given by:

$$E_n(x) = \int_1^\infty e^{-xu} u^{-n}\, du$$

Legends for Illustrations on Opposite Page

Fig. 15—Characteristics of epilated skin lesions in sheep made by beta irradiation of sheared areas (a, c, and e) and of areas covered by 33 mm. of growing wool (b, d, and f).

a) The most atrophic portion of a lesion, 33 days after 15,000 rep of beta radiation. x 56.

b) The most atrophic area from the epilated area, caused by 16,000 rep delivered to the surface of the wool. Epilation has resulted in the latter case from partial atrophy of follicles without apparent effect on the other structures, and regrowth of wool would have occurred normally in this area. Except for remnants of muscles, no remnants of other appendages are present in a, and epilation would have been permanent. x 56.

c) The persistent active ulceration in the center of a lesion produced 33 days previously by exposure of sheared skin to 25,500 rep of beta radiation. Acanthotic islands of abnormal epidermis are present in loose areolar tissue heavily infiltrated with lymphocytes. Muscle and sweat glands can be seen below the exudate. Part of the scab above the lesion is also present. x 86.

d) In contrast, this is the final appearance of the skin after 145,000 rep of beta radiation to the surface of growing wool. There has been no effect upon the deep reticular layer of the dermis. The more superficial papillary layer is almost completely atrophic. A large arrectores pilorum muscle is seen remaining in it just above several arterioles. x 86.

e) The edge of the ulcer in c. x 86.

f) The edge of atrophic crater in d. These two examples of epidermal and follicular hyperplasia are seen to be unaccompanied by hyperplasia of the sebaceous glands. In the natural lesion of the ears of the sheep dying in Utah, the epidermal hyperplasia was accompanied by a relative hyperplasia of the sebaceous glands, as seen in figure 12d. x 86.

When, as in the present case, sec θ is only slightly greater than unity, the ratios can be calculated more easily from the right-hand sides of the equations above. The calculated ratios, expressed as percentages, are shown in table 3. These data show that the ratio of wool surface dose to skin dose is not very sensitive to the uncertain factors and assumptions used in the calculation and the average theoreti-

TABLE 3—The Percentage of the Dose at the Surface of the Beta Applicator Reaching the Skin 3.3 cm. Below the Surface of the Wool

Assumed applicator filtration (mg./cm.²)	Attenuation coefficients[10, 11]			
	2.3 cm.²/Gm.		7.8 cm.²/Gm.	
	Thin (%)	Thick (%)	Thin (%)	Thick (%)
50	1.5	3.5	2.0	3.7
100	1.9	4.0	3.0	4.7
200	2.8	4.9	4.9	6.7
400	4.3	6.5	8.4	10.3

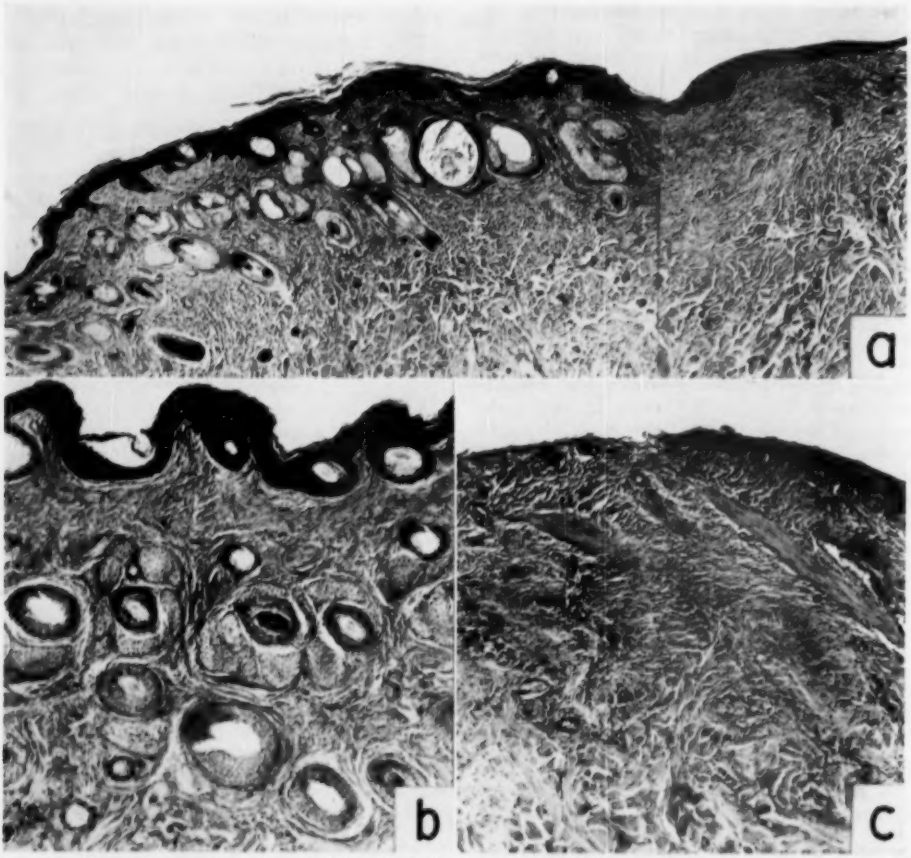

Fig. 16—Photomicrographs of typical histological features of a dermal ulceration caused by known accidental beta irradiation of the skin of a horse.

a) A panoramic view of the lesion proceeding from left to right from relatively normal follicles and epidermis to an area of complete atrophy of dermal structures. In the center are large follicular cysts occurring in atrophic follicles. Notice the isolated persisting sebaceous gland at the edge of the atrophic area. x 67.

b) Sebaceous glands exhibiting little evidence of atrophy among hair follicles and showing various stages of atrophy. x 102.

c) Persistent arrectores pilorum muscles exhibiting no noticeable effect of the radiation which has caused the complete loss of all other structures in the area and the ulceration of the epidermis above them. x 102.

cally derived dose is consistent with the biological observations.

SUMMARY OF CONCLUSIONS

1) The skin lesions of Utah range sheep, originally thought to be from fall-out contamination by *beta* ray-emitting substances, were a fulminating variant of the naturally occurring disease called infectious pustular dermatitis.

2) The hit-or-miss destruction of the dermal structures by this infection and the failure of the arrectores pilorum muscles to persist prove that the lesions were not the result of radiation damage.

3) The threshold dose for epilation of the sheared sheep skin lies between 2,500 and 5,000 rep.

4) The threshold dose for epilation of the normally wooled skin of the face lies between 5,000 and 10,000 rep.

5) The threshold dose for epilation of a thickly wooled sheep (staple length of 33 mm.) is probably between 10,000 and 15,000 rep.

6) The amount of *beta* irradiation that would have been required to produce lesions as extensive as those caused by the infectious pustular dermatitis is estimated to be between 50,000 and 150,000 rep.

7) Sheep are naturally well protected from *beta* radiation damage by the thickness of their wool.

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In Vitro Propagation of Hog Cholera Virus. I. Method of Cultivation and Observations on Color Changes in the Medium

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PROPAGATION of hog cholera virus *in vitro* was first reported in 1932 by Hecke.[1] Subsequently, reports of successful propagation *in vitro* were made by TenBroeck,[2] in 1941; by Boynton,[3] in 1946; and by Frenkel and van Bekkum,[4] in 1953. A second report was made by Frenkel *et al.*[5] in 1955. In all of these reports, the authors described their methods and reported on the quantities of virus obtained. Hecke [1] obtained infectivity titers of 10^{-5}. TenBroeck [2] demonstrated infectivity titers of 10^{-9} in swine. In an infectivity titration experiment by Boynton,[3] the seventh generation of virus propagated in tissue culture produced cholera in 2 pigs, each inoculated with 2 cc. of a dilution of 1×10^{-9}, the highest dilution tested.

Extensive infectivity titrations with virus blood from pigs injected with hog cholera virus have been reported by Cole *et al.*[6] and Cole and Henley.[7] The first authors [6] concluded that a minimum lethal dose between 1/1,000,000 cc. and 1/2,500,000 cc. was indicated from their results. Cole and Henley [7] occasionally produced cholera by doses as small as 1/5,000,000 cc., which was usually the highest dilution they tested. Schwarte and Mathews [8] reported viruses which produced hog cholera in dilutions varying from 10^{-3} to 10^{-6}. They described this variation in titer as a characteristic of different strains. In all these reports often only 1 pig, but not more than 2, was injected with each dilution.

By the use of larger numbers of animals injected with each dilution, even though limited to two dilutions, it would seem to be possible to arrive at a much closer determination. However, even with such a procedure the cost of animals and isolation barns for this purpose is excessive.

With increased attention being given to the propagation of hog cholera virus *in vitro* and its use in the production of some immunizing agents, the need for knowledge of a more exact quantity of virus entering into such products is increased in order, perhaps, to correlate this factor with the degree of immunizing value of the product. It would seem that if, under seemingly duplicate procedures, one product is prepared from several times more virus than another, the degree of immunizing value of these products may vary appreciably, even though it has been reported [9] that no correlation was found between the titers of blood viruses and the potencies of the derived vaccines.

This report describes (1) the method used to establish propagation of hog cholera virus in mediums with explants of swine spleen and (2) the *in vitro* method used for the titration of an agent multiplying concomitantly with the hog cholera virus.

MATERIALS AND METHODS

For propagating the hog cholera virus, the method as described by the Netherlands workers [4, 5] was followed as closely as possible. However, a number of unavoidable modifications were necessary, the significance of which, if any, has not yet been evaluated.

For the routine passage of the hog cholera virus, 500-cc., wide-mouthed Erlenmeyer flasks were used initially. They were each fitted with a No. 10 two-hole rubber stopper, carrying two glass tubes 8 mm. in diameter. One tube was bent at a slight angle just below the stopper so that the end of the tube came within 1 or 2 mm. from the bottom of the flask at the periphery. Aeration of the medium was provided through this tube. The second tube extended an inch below the stopper and served as an escape for the gas introduced. Both tubes were bent at right angles about an inch above the stoppers. The two tubes were fitted with cotton plugs as filters.

A rotary shaker was used to supply constant agitation with rotations of 125 per minute. The agitation provided repeated exposure of the liquid portion of the medium to the atmosphere within the flask.

Ordinarily, two serial passages were made each week, one being incubated for three days and one

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for four days. All cultures were incubated at 37.5 C.

For the method of assaying *in vitro* the agent multiplying concomitantly with the hog cholera virus, 250-cc. Erlenmeyer flasks were used. They were fitted with No. 6 rubber stoppers that carried equipment like that of the larger flasks except that 6-mm. glass tubing, with correspondingly smaller rubber connections, was used. In these, the glass tubing conveying the carbogen* was not bent and the gas was released at the center of the bottom of the flask. The assembly of equipment is shown (fig. 1).

Swine spleen was used to provide the living cells for the culture medium. The spleen was removed aseptically at the time of slaughter, collected in Hanks' salt solution,[10] and stored at 8.0 C. until used on the same day in the preparation of the cultures. The spleen was minced with hand scissors into pieces approximately 1 mm. in diameter. Later, a section of spleen about 1 cm. in diameter was added at times to provide material for histological examination.

The pigs serving as the source of the spleens and those used for the infectivity tests were obtained at weaning time from the herd at the Agricultural Research Center, Beltsville, Md. The ages of the pigs at the time of use varied from 70 days to 6 months.

Preparation of Culture Medium.—The culture medium, which was essentially a modified Tyrode solution, was prepared as that used by the Netherlands workers.[4,6] The composition of the medium prepared for incubation in a 500-cc. flask was as follows:

Tyrode-cystine-peptone (TCP) fluid	110.0 cc.
Swine serum (normal)	60.0 cc.
$NaHCO_3$ (1.4%)	20.0 cc.
Minced spleen tissue	10.0 Gm.
Virus inoculum	10.0 cc.
Penicillin	300.0 units/cc. of fluid
Streptomycin	50.0 μg./cc. of fluid

The composition of the TCP fluid was as follows:

NaCl, Merck, ACS	7.9 Gm.
KCl, Merck, ACS	0.2 Gm.
$CaCl_2$, Merck, ACS	0.263 Gm.
$MgCl_2$ $6H_2O$, Merck, ACS	0.1 Gm.
NaH_2PO_4 anhydrous, Merck, ACS	0.044 Gm.
$NaHCO_3$, Merck	1.0 Gm.
Dextrose anhydrous, Merck	2.0 Gm.
Bacto-peptone, Difco	3.0 Gm.
l-cystine HCl, Merck	0.125 Gm.
Distilled H_2O	1,000.0 cc.

The $NaHCO_3$ for the TCP fluid was prepared separately as a 5.0 per cent solution in distilled water, autoclaved at 15-lb. pressure for 30 minutes, and cooled before adding to the other components of the fluid. The other nutrient constituents of the TCP fluid, as well as the 1.4 per cent solution of $NaHCO_3$ used in the medium itself, were sterilized in the same manner as the 5.0 per cent $NaHCO_3$ solution.

The swine serum was filtered through a No. 9 Mandler candle and stored at 8.0 C. The age of the serum when used in the medium varied from one to 20 days.

The minced spleen tissue was mixed with the 10.0 cc. of virus used as the inoculum, and the mixture was held at 8.0 C. for an hour before being added to the other constituents of the culture.

The culture fluid for the 250-cc. flasks for titrations was used in one-fourth the quantity used in the 500-cc. flasks. After incubation, tenfold serial dilutions for inoculations were prepared with phosphate buffer with a pH of 7.4. Each of the flasks was seeded with 1.0 cc. of the dilutions. Unless otherwise stated, the same amount of these or similar dilutions was injected into separate pigs whenever animal tests were used for infectivity titrations.

Each pig used in titration tests was isolated in a separate pen. The pigs were fed and watered over the lower half of a two-section door so that the attendant did not enter the pens of the normal animals. In all cases, surviving animals were shown to be susceptible to cholera upon subsequent challenge.

All cultures were routinely tested for bacterial contamination.

The mediums were not routinely tested for pH values either before or after incubation. However, when tested, the mediums, just prior to the beginning of incubation, had pH values varying from 8.0 to 8.5. At the time of harvest the pH varied from 7.2 to 7.6.

Procedure for Harvesting Virus.—After incubation, the supernatant fluid of the culture medium was decanted into a separate flask and 100 cc. of buffered saline was added to the residue. Alternate treatments of one minute each in a Waring blendor and an ice-water bath repeated five times were made before returning the tissue suspension to the flask containing the supernatant fluid of the tissue culture. Quantities in amounts of 35 cc. were partially cleared by centrifugation in conical centrifuge tubes at 2,500 r.p.m. for 15 minutes in an International No. 1 centrifuge, with a 240 A head. A portion of the supernatant was set aside as the inoculum for the succeeding passage. Other portions in amounts of 12 cc. were bottled and stored in a chest of dry ice for a source of needed virus.

Method of Scoring Titration Reactions of the Agent Discoloring the Medium.—As the routine passages were made, visible changes in the physical characteristics of the cultures became evident. At first, it appeared that these changes occurred in the cultures where hog cholera virus was propagating but did not occur, or occurred to a much lesser extent, in control flasks. The most noticeable differences were a change in color of the fluid of the culture and a change in color of the

* A mixture of 95 per cent oxygen and 5 per cent carbon dioxide.

tissue. Since these changes were largely of degree, the extent when compared with the control was recorded as follows:

0 = comparatively no change; slight grayish color of fluid; less than 10 per cent of tissue showing loss of red color.
+ = slight grayish color of fluid; 10 to 25 per cent of tissue showing loss of red color.
++ = noticeable grayish color of fluid; 25 to 50 per cent of tissue showing loss of color.
+++ = marked grayish color of fluid; 50 to 75 per cent of tissue showing loss of color.
++++ = intense grayish color of fluid; 75 to 100 per cent of tissue showing loss of color.

Virus 2266.—This was the ninth serial passage in swine of BAI virus 276, which was described by Dale *et al.*[12]

Vaccines.—Crystal-violet vaccines were prepared according to the method described by Cole and Henley,[11] with modifications associated with the different vehicle for the hog cholera virus.

RESULTS

The first passage of the first series (A) was made routinely with 10 cc. of sterile serum virus 2266, as the original inoculum. This passage was the only one made without shaking and, when examined on the third day of incubation, it was noted that the rubber stopper had been ejected from the mouth of the flask. Gas had been introduced at such a rate that the cotton in the escape tube had been soaked with fluid carried by the gas bubbles. The soaked cotton prevented the escape of gas and, as a result, enough pressure accumulated in the flask to blow out the rubber stopper. Because of these irregularities, a new series (B) was started at the same time as the second passage of series A, since it seemed possible that the virus used to inoculate the first passage may not have survived.

When the third passage of A and the second passage of B were harvested, it was noted that the A culture was redder than the B culture. Each of the cultures in amounts of 2 cc. was injected into pigs. As anticipated, the series B culture produced cholera but the series A culture did

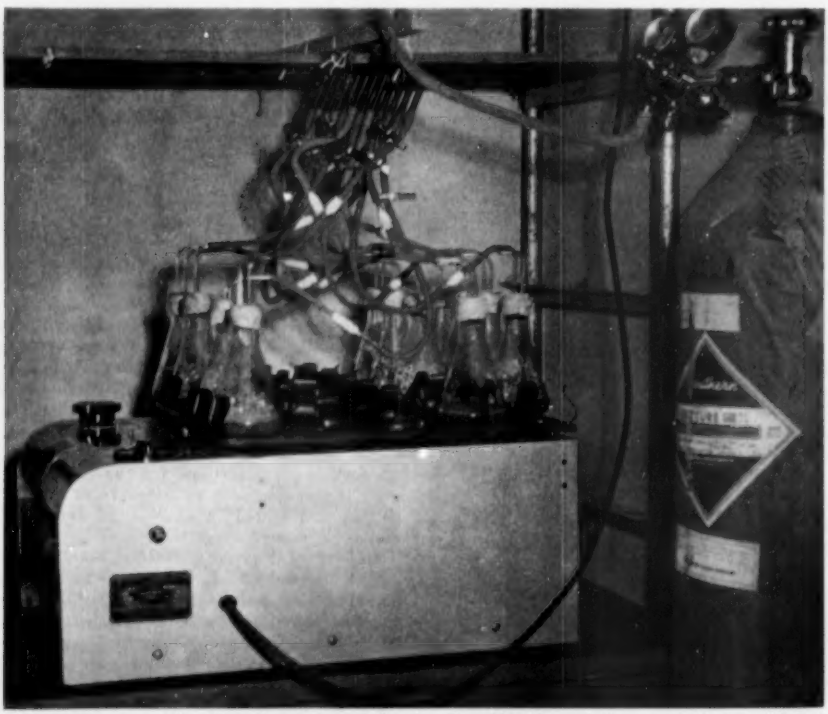

Fig. 1—The assembly of equipment used for in vitro titration of the agent that multiplied concomitantly with hog cholera virus.

not. These results suggested that the discoloration of the medium of series B might be an indication of virus propagation. Therefore, it was decided to continue the series A passages as a control.

The sixth passage of A and the fourth passage of B, incubated simultaneously, were tested for virus by pig injections. Again, the B culture produced cholera while the A culture did not.

As the two series of passages were continued, the discoloration in series B persisted. In the later passages, the change in color appeared sooner than in the earlier ones, being very noticeable within 48 hours and reaching its height by 72 hours.

When this discoloration began to appear in the eighth passage of series A, it seemed that this characteristic was not associated with the propagation of hog cholera virus. Because of this view, a new control series (C) without virus was started.

When the mediums of series C failed to become discolored by the third passage, a titration test in tissue culture mediums of the tenth passage of series B was made. Dilutions of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} were prepared and injected into pigs in 2-cc. amounts, using 2 pigs for each dilution. Those injected with dilutions 10^{-4} died of hog cholera, while those injected with higher dilutions remained well.

At the same time the pigs were injected, four flasks were seeded with the 10^{-4} through the 10^{-7} dilution. At the end of five days' incubation, all four tissue cultures were discolored. The daily changes are shown (table 1). After five days of incubation, fluid from each culture was preserved by freezing and was stored in a chest of dry ice. When injected into pigs 14 days later, none of these fluids produced hog cholera. These negative results indicated that the agent being propagated and causing the discoloration was not hog cholera virus.

In the meantime, a titration test in tissue culture of the twelfth passage of series B was made. The test was also designed to find out whether or not there would be a difference between shaking the tissue culture with carbogen and shaking it without carbogen. Four dilutions of the culture, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, were compared. Two flasks of culture, one seeded with 1 cc. of undiluted virus and one unseeded, were included as controls.

TABLE 1—Titration in Vitro to Determine the Value of Discoloration of a Medium of Explanted Tissue as an Indicator of Hog Cholera Virus Multiplication

Dilution of 10th passage of hog cholera virus[1]	Components of medium	Hours of incubation			
		48	72	96	120
10^{-4}	Fluid	[2]0	2+	4+	[3]4+
	Tissue	0	0	3+	4+
10^{-5}	Fluid	0	1+	2+	[3]3+
	Tissue	0	0	2+	3+
10^{-6}	Fluid	0	0	1+	[3]3+
	Tissue	0	0	1+	2+
10^{-7}	Fluid	0	0	1+	[3]2+
	Tissue	0	0	1+	2+

[1] This passage produced hog cholera in pigs inoculated with 2 cc. of the 10^{-4} dilution but did not produce hog cholera with the 10^{-5} through the 10^{-7} dilution. Of the 2 pigs inoculated with the 10^{-4} dilution, 1 died on the seventh day and the other on the twelfth day after inoculation. At necropsy, both showed lesions commonly associated with hog cholera.

[2] 0 to 4+ = degree of discoloration.

[3] This fluid did not produce hog cholera.

TABLE 2—Effects of Carbogen and Shaking (Aeration) on Titration in Vitro of Agent that Multiplied Concomitantly with Hog Cholera Virus in a Medium of Explanted Tissue

Treatment	Components of medium	Hours of incubation for different dilutions and controls																			
		Dilutions[1]												Controls							
		10^{-4}			10^{-5}			10^{-6}			10^{-7}			Undiluted virus			No virus				
		48	72	88	48	72	88	48	72	88	48	72	88	48	72	88	48	72	88		
Carbogen and shaking	Fluid	[2]4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	4+	[3]4+	3+	4+	3+	2+	2+	[3]2+		
	Tissue	3+	4+	4+	3+	4+	4+	2+	4+	4+	1+	3+	3+	3+	4+	4+	1+	1+	2+		
Shaking without carbogen	Fluid	2+	2+	3+	2+	2+	3+	2+	2+	3+	1+	2+	[3]3+	....	....	....	....	....	....		
	Tissue	1+	2+	3+	1+	2+	3+	0	1+	3+	0	1+	3+	....	....	....	....	....	....		
Without shaking or carbogen	Fluid	1+	1+	[3]1+	1+	1+	1+	....	....	....	....	....	....	....	....	....	....	....	....		
	Tissue	0	0	0	0	0	0	....	....	....	....	....	....	....	....	....	....	....	....		

[1] This was the twelfth passage *in vitro* of hog cholera virus. It was not tested in pigs. The eleventh and thirteenth passages produced hog cholera.

[2] 0 to 4+ = degree of discoloration.

[3] This fluid did not produce hog cholera.

In addition, two flasks of culture, one seeded with a 10^{-4} dilution, the other with 10^{-5}, were not shaken or treated with carbogen. The results of this test are shown (table 2).

The first observation was made at 48 hours of incubation. At this time, the three lowest dilutions treated by shaking with carbogen showed definite discoloration and there was some indication that the 10^{-7} dilution would be discolored. In the flasks of culture which were shaken with no carbogen, there was no difference readily detectable by visual observation between the flasks seeded with the 10^{-4} and 10^{-5} dilutions and the negative control flask treated with shaking and carbogen. The mediums in the flasks seeded with the 10^{-6} and 10^{-7} dilutions were even less discolored.

At the 72- and 88-hour periods of incubation, the degree of intensity of discoloration between the treatment of shaking with carbogen and without carbogen was more easily readable. Incubation was terminated after 88 hours.

On the day following the incubation period, fluids from the two flasks seeded with the 10^{-7} dilutions, from the flask not shaken and without carbogen seeded with the 10^{-4} dilution, and from the negative control flask were injected into separate pigs. These fluids had been held in a chest of dry ice. These 4 pigs were injected on the same day as the 4 pigs referred to above as having been injected with the tenth-passage fluids collected after five days of incubation.

When none of these 8 pigs developed cholera, it was concluded that the discoloration of the mediums was due to the multiplication of some agent other than hog cholera virus.

Before the results of the injections of these 8 pigs were known, a titration test in tissue culture medium of the thirteenth passage of series B was made. Four flasks were seeded with the 10^{-7} dilution, four flasks with the 10^{-8} dilution, and two flasks with the 10^{-9} dilution. Two control flasks were used—one seeded with undiluted inoculum and the other unseeded. The results of this titration are given (table 3). Calculated according to the method of Reed and Muench,[12] the 50 per cent positive end point (i.d.$_{50}$) was $10^{-8.5}$.

After incubation of the cultures for 72 hours, separate pigs were injected with fluids from two flasks seeded with 10^{-8} dilutions, one of which showed positive and one of which showed negative discoloration, and fluid from the flask seeded with undiluted culture. At the same time, separate pigs were injected with undiluted fluids from the seventh, eleventh, and thirteenth passages. These latter 3 pigs and the 1 injected with the culture from the flask seeded with undiluted inoculum from the thirteenth passage succumbed to hog cholera. Typical courses of hog cholera occurred in each case. The pigs injected with the 10^{-8} dilution cultures remained normal.

Preparation of Stock Supply of Hog Cholera Virus Propagated in Tissue Culture.—In order to make further observations on the discoloration being obtained in the tissue cultures and to continue the hog cholera virus propagation, a stock supply of hog cholera virus was prepared. A routine passage of the thirteenth serial passage of B was made. After three days'

TABLE 3—Titration in Vitro of Agent that Multiplied Concomitantly with Hog Cholera Virus in a Medium of Explanted Tissue

Dilution of 13th passage of hog cholera virus [1]	Components of medium	Hours of incubation in flasks								Results [2]
		Flask 1		Flask 2		Flask 3		Flask 4		
		64	72	64	72	64	72	64	72	
10^{-7}	Fluid	[3]3+	4+	3+	4+	3+	4+	3+	4+	4/4
	Tissue	3+	4+	3+	4+	3+	4+	3+	4+	
10^{-8}	Fluid	3+	4+	3+	4+	1+	1+	3+	4+	3/4
	Tissue	2+	3+	2+	3+	1+	1+	3+	3+	
10^{-9}	Fluid	1+	1+	1+	1+	....	....	....	....	0/2
	Tissue	0	0	0	1+	....	....	....	....	
Positive control (1 cc.)	Fluid	4+	4+	4+	4+	....	....	....	....	2/2
	Tissue	4+	4+	4+	4+	....	....	....	....	
Negative control (1 cc.)	Fluid	1+	1+	....	....	....	....	....	....	0/2
	Tissue	1+	1+	....	....	....	....	....	....	

[1] This passage produced hog cholera in a pig inoculated with 1 cc. of the undiluted culture.
[2] The denominator indicates number of flasks seeded and the numerator indicates number positive.
[3] 0 to 4+ = degree of discoloration.

incubation, this was harvested and stored in amounts of 12 cc. as a primary source of virus. One of these samples was used before freezing to make the next serial passage, the fifteenth. This passage was harvested after four days' incubation, frozen, and stored in amounts of 12 cc. The latter supply represented stock virus.

A titration *in vitro* was made of the agent multiplying in the stock culture (15th passage) with hog cholera virus. This passage was also titrated for hog cholera infectivity in pigs. The results of both titrations are given (table 4). The medium of only one of the four flasks seeded with the 10^{-5} dilution was discolored. The results of this titration gave a positive 50 per cent end point of $10^{-7.6}$. They also show, as in previous tests, that the agent which multiplied concomitantly with the hog cholera virus also multiplied in the absence of hog cholera virus, since the medium of the negative control flask, which was not inoculated with hog cholera virus, was as discolored as the mediums which contained hog cholera virus.

In this test, this agent was so established after seven serial passages that it produced as marked discoloration of the fluid and tissue of the medium in the absence as it did in the presence of hog cholera virus.

Results of Tests for Bacterial Contamination and Pathogenicity.—All tests for bacterial growth were consistently negative. No evidence or pathogenicity for mice or guinea pigs was detected. The undiluted culture was injected intracranially, intraperitoneally, intramuscularly, and subcutaneously in mice and intramuscularly and subcutaneously in guinea pigs.

Results with Vaccines.—Crystal-violet vaccines were prepared from the tenth, eleventh, and twelfth passages of hog cholera virus. In 10-cc. amounts, they failed to produce any evidence of immunity in pigs when they were subsequently challenged with regular hog cholera virus.

DISCUSSION

Little difficulty was experienced in establishing *in vitro* propagation of hog cholera virus by methods described by Frenkel *et al.*[5]

The minced spleen added to the medium gave a reddish color to the fluid. This color was undoubtedly due to the freed red blood cells concentrated in the normal tissue. During the first few passages, this color of the medium, as well as the reddish color of the tissue, was maintained throughout the entire incubation periods. However, as the passages were continued, the fluid medium as well as the tissue took on a grayish color. It is generally accepted that this change in color is associated with destruction of hemoglobin. Since this discoloration occurred more or less concurrently with the propagation of hog cholera virus, it appeared at first

TABLE 4—Titration in Vitro of Agent that Multiplied Concomitantly with Hog Cholera Virus in a Medium of Explanted Tissue

Dilution of 15th passage of hog cholera virus[1]	Components of medium	Hours of incubation of flasks																Results[3]
		Flask 1				Flask 2				Flask 3				Flask 4				
		24	48	72	120	24	48	72	120	24	48	72	120	24	48	72	120	
10^{-7}	Fluid	[2]0	0	[4]3+	....	0	0	3+	....	0	0	3+	....	0	0	3+	....	4/4
	Tissue	0	0	3+	....	0	0	3+	....	0	0	3+	....	0	0	3+	....	
10^{-6}	Fluid	0	0	+	+	0	0	[4]3+	....	0	0	+	+	0	0	+	+	1/4
	Tissue	0	0	±	+	0	0	3+	....	0	0	±	+	0	0	±	+	
10^{-5}	Fluid	0	0	+	+	0	0	+	+	....	....	....	....	....	....	....	....	0/2
	Tissue	0	0	±	+	0	0	±	+	....	....	....	....	....	....	....	....	
Positive control (1 cc.)	Fluid	2+	3+	4+	4+	....	....	....	....	....	....	....	....	....	....	....	....	1/1
	Tissue	0	1+	4+	4+	....	....	....	....	....	....	....	....	....	....	....	....	
Negative control (1 cc.)[5]	Fluid	2+	3+	4+	4+	....	....	....	....	....	....	....	....	....	....	....	....	1/1
	Tissue	0	1+	4+	4+	....	....	....	....	....	....	....	....	....	....	....	....	

[1] This passage produced hog cholera in pigs inoculated with the 10^{-2} through the 10^{-4} dilution but did not produce hog cholera in the 10^{-5} through the 10^{-6} dilution. Four of the 6 pigs died in seven to ten days; the other 2 were killed on the tenth day when 1 was moribund and the other 1 showed signs of advanced hog cholera.

[2] The denominator indicates the number of flasks seeded and the numerator indicates the number positive.

[3] 0 to 4+ = degree of discoloration.

[4] Incubation of the four 10^{-7} flasks and the positive 10^{-5} flask was discontinued at 72 hours.

[5] This was the sixth passage of control series C.

that there was a direct relationship between these two biological activities. Because of the limited space for housing swine individually for titrations for hog cholera virus, weeks were required before the necessary animal inoculations were completed. Since the *in vitro* titrations of the agent associated with the discoloration of the medium required only a few days, it was possible to complete several of these *in vitro* tests before establishing that there was no direct correlation between the two actions. However, it has not yet been determined whether or not the presence of this agent has any effect on the propagation of the hog cholera virus.

SUMMARY

In vitro propagation of hog cholera virus was readily established in a fluid medium (modified Tyrode's) containing minced tissue of swine spleen. By serial passages of three or four days' incubation, the fifteenth passage gave a 10^{-5} infectivity titer when tested in swine. Dilutions of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} were injected into pigs, using 2 pigs for each dilution. Those pigs receiving 10^{-5} dilutions or lower died from hog cholera, while those receiving higher dilutions remained normal.

Crystal-violet vaccines separately prepared with virus from the tenth, eleventh, and twelfth passages failed to produce any detectable degree of immunity in swine given subcutaneous injections of 10 cc.

An agent which produced discoloration of the medium was propagated concomitantly with the hog cholera virus. By *in vitro* titration, it was shown to have a titer varying from $10^{-7.6}$ to $10^{-8.3}$. A correlation between the titers of the two propagated agents was not demonstrated.

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Bromsulphalein Clearance in the Horse—A Quantitative Liver Function Test

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THE ABILITY of the liver to selectively excrete foreign dyes introduced into the general circulation has long been accepted as one of the more satisfactory liver function tests in human and canine medicine. No single test for liver function is universally accepted, since the organ in various disease conditions may lose specific biochemical functions in differing orders. This dissociation of liver function has continually challenged the clinical investigator. Rose bengal[1,2] and bromsulphalein® (BSP)[3] have been used experimentally in ruminants to assay liver function, with sampling of the serum at 15 and 45 minutes for dye retention. It was concluded from these studies that the test, as conducted, was dependable only in the late stages of diffuse fibrosis of the liver as in Senechio poisoning or severe fluke infection. Others concluded that the dye-excretion tests were of no use in the diagnosis of ruminant hepatopathy. Data from our laboratory indicate that this test, with certain modifications, can be used to obtain a quantitative assessment of liver function in large animals. This report is confined to the equine species.

MATERIALS AND METHODS

Control horses were between the ages of 4 and 20 years and of both sexes. Testing was done under standard conditions that consisted of (1) absence of jaundice, (2) absence of fever, (3) a normal appetite, and (4) the maintenance of inactivity during the test.

All blood samples were collected via the left jugular vein following the injection of 1 Gm. of BSP* into the right jugular vein. Polyethylene medical tubing was inserted into the veins of hypersensitive horses in which sampling by multiple venipuncture would produce excitation. Injection of the dye required between 15 and 30 seconds and the mean injection time was used as the zero time. Frequent, accurately timed blood samples were obtained for a 30-minute period in most cases. Since BSP[4] becomes bound to the plasma proteins, samples were taken 4.5 minutes after the injection of dye to allow for mixing. Each heparinized blood sample (8 to 10 ml.) was centrifuged and 2 ml. of plasma was transferred to a 19- by 105-mm. cuvette containing 3 ml. of 0.1 N NaOH and 1 ml. of water. The blank consisted of 2 ml. of plasma, 3 ml. of 0.1 N HCl, and 1 ml. of water. A Coleman junior spectrophotometer model 6A was used for preparation of the standard curve at a wave length of 565 mμ.

Biliary BSP samples were diluted with 0.1 N NaOH until accurate absorption limits of the spectrophotometer were obtained. Preinjection bile at an equal dilution with the postinjection bile served as a blank since bile samples became turbid upon the addition of dilute HCl.

The plasma dye concentration (P) is plotted as its logarithm against time (t) in minutes (fig. 1). The initial dye concentration can be estimated from the intercept of the extrapolated line with the ordinate at zero time.[4] The plasma volume has been previously demonstrated to be equal with the

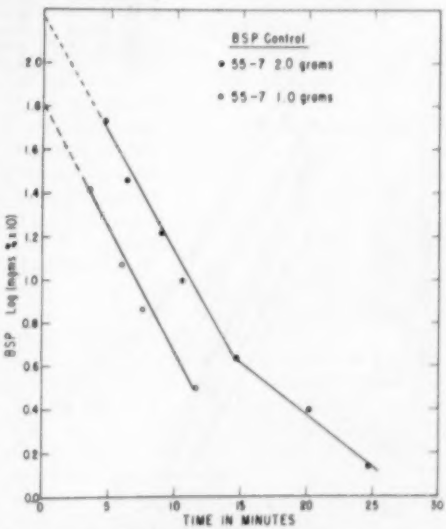

Fig. 1—Bromsulphalein (BSP) clearance in horse 55-7 following the administration of 1 and 2 Gm. of BSP (one-week interval).

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The authors are deeply indebted to R. W. Brauer of the U. S. Naval Radiological Defense Laboratory, San Francisco, Calif.

* This material was made available through the courtesy of H. B. Dunning of Hynson, Westcott and Dunning, Inc., Baltimore, Md.

BSP pool.[5,6] The volume (V) into which the dye was diluted can be calculated, since the initial concentration (Xo), the number of milliliters of dye (i) injected, and its concentration (Xi) are known.

$$V = \left(\frac{Xi}{Xo} - 1 \right) i$$

The slope (k) of the semilog plots as shown (fig. 1 and 2) is proportional to the fractional clearance K or $\frac{c}{V}$ [5,6] (K = 2.3 k, and 2.3 log a = 1n a).

$$K = \frac{c}{V} = 2.3 \times \frac{\log P1 - \log P2}{t2 - t1}$$

Half-time ($t_{1/2}$) values for dye clearance are easily calculated if fractional clearance (K) is known.[7]

$$t_{1/2} = \frac{1n\ 2}{K} \qquad 1n = \text{natural log}$$
$$1n\ 2 = 0.692$$

Furthermore, fractional clearance $\frac{c}{V}$ multiplied by the BSP pool (V) provides the clearance (c) in milliliters of plasma per minute. From figure 1, sample calculations were made following the injection of 1 Gm. of BSP (3.2 mg./kg.).

Anilog 1.80/10 = 6.3 mg./100 cc.

$$V = \left(\frac{50}{0.063} - 1 \right) 20 = 15.8 \text{ liters}$$

$$k = 1.30/11.9 = 0.109$$

$$K = 0.109 \times 2.3 = 0.251$$

$$c = 0.251 \times 15.8 = 3.98 \text{ liters plasma per minute}$$

$$t_{1/2} = \frac{0.692}{0.251} = 2.75 \text{ minutes}$$

Calculations from the 2-Gm. injection (6.3 mg./kg.) indicated in the same horse, seven days later, a BSP pool of 16.9 liters, a K value of 0.237, and a $t_{1/2}$ of 2.5 minutes. Known standard values for horses[8] are 51 ml. of plasma per kilogram of body weight. Horse 55-7, at 315 kg. of body weight, should by these standards have 16.0 liters of plasma which is in fairly close agreement with both levels of BSP administration.

Two horses received 250 ml. of carbon tetrachloride (CCl_4) and 500 ml. of mineral oil by stomach tube. The CCl_4 mixture was administered in 3 gal. of water. One horse (55-8) received 250 Gm. of glucose intravenously at the time of the CCl_4 administration.

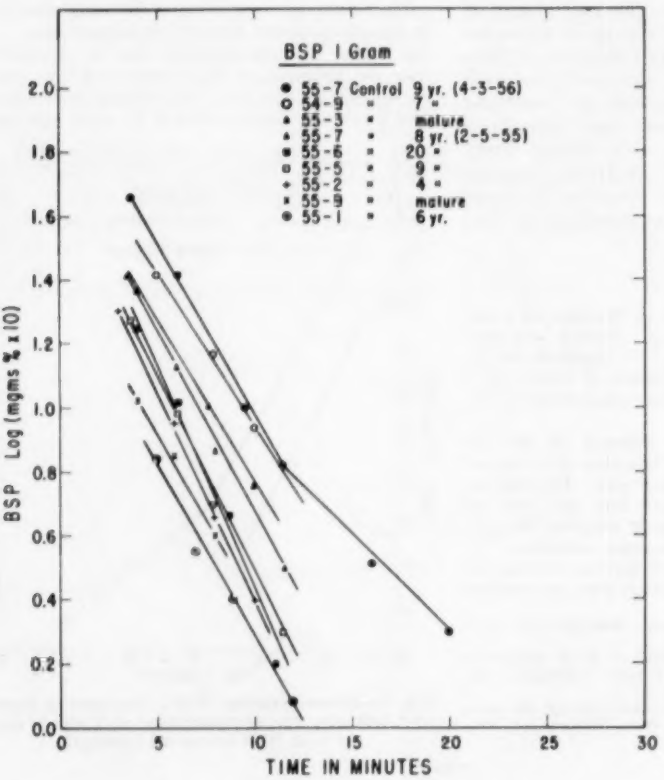

Fig. 2—Bromsulphalein (BSP) clearance in horses of various ages following the administration of 1 Gm. of BSP.

RESULTS

Mean fractional clearance (K) for 14 control horses using 1 Gm. of BSP was 0.255 ± 0.05, with a range of 0.186 to 0.36 for the first 12 minutes. Mean half-times ($t_{1/2}$) were 2.81 ± 0.5, with a range of 2.0 to 3.7 minutes (fig. 2). This exponential BSP clearance was not altered appreciably in the same horse by increasing the quantity of dye injected as high as 6.35 mg./kg. (fig. 1). The same horse (55-7), tested at yearly intervals under different weight conditions, had nearly identical fractional clearance values. Following the first 12 minutes, a slower exponential clearance is observed when sufficient dye remains for detection. A biliary fistula preparation was undertaken to find if the enterohepatic circulation of BSP could be partly involved in producing this second slower component of dye clearance. In the first 12 minutes following intravenous injection, the mean concentration was 0.1 mg. of BSP per milliliter of bile. In the second bile aliquot (12 to 20 minutes), a mean concentration of 2.0 mg. of BSP per milliliter was observed, with 25 per cent of the total BSP injected appearing in the bile within the first hour. Because of the absence of a gallbladder, the continuous secretion of bile allows for a short time lag between liver acceptance of the dye and its excretion. Unpublished data from our laboratory indicate that nearly 90 per cent of the injected dose of dye can be recovered from the hepatic duct bile of control sheep in the first 4.5 hours, followed by smaller amounts thereafter. Extrahepatic uptake of BSP would appear to be negligible under such conditions.

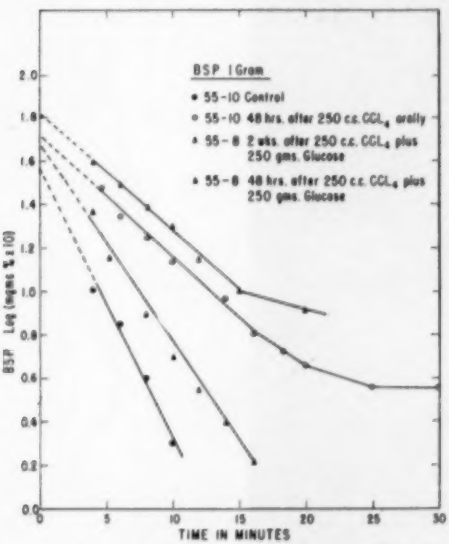
BSP 1 Gram
• 55-10 Control
• 55-10 48 hrs. after 250 c.c. CCl4 orally
▲ 55-8 2 wks. after 250 c.c. CCl4 plus 250 gms. Glucose
▲ 55-8 48 hrs. after 250 c.c. CCl4 plus 250 gms. Glucose
BSP Log (mgms % x 10)
TIME IN MINUTES

Fig. 3—Bromsulphalein clearance in horses before and after CCl_4 administration.

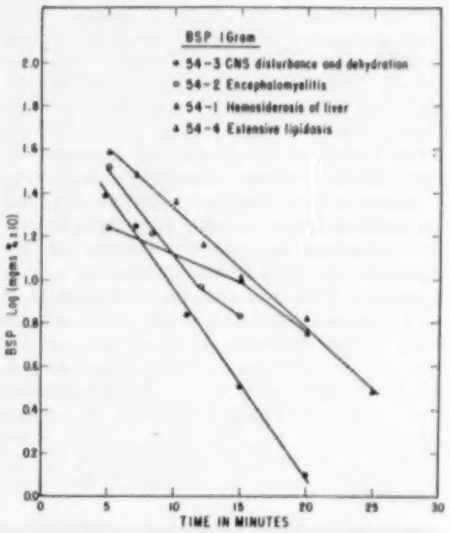
BSP 1 Gram
• 54-3 CNS disturbance and dehydration
○ 54-2 Encephalomyelitis
▲ 54-1 Hemosiderosis of liver
▲ 54-4 Extensive lipidosis
BSP Log (mgms % x 10)
TIME IN MINUTES

Fig. 4—Bromsulphalein clearance in hospitalized animals. Note normal clearance in horses 54-2 and 54-3 which ruled out hepatolenticular degeneration.

Two horses, 48 hours following administration of 250 ml. of CCl_4, exhibited fractional clearances of 0.128 and 0.111 in the first 12 minutes of dye disappearance (fig. 3). Following the first 12 minutes, even lower fractional clearances were observed. The inability of the liver to exerete BSP into the bile as fast as it is being cleared from the blood could account partly for this decreased fractional clearance. Horse 55-8 exhibited normal clearance values two weeks after having received the CCl_4. Horse 55-10 was killed immediately following the BSP clearance test 48 hours after CCl_4 administration. There was marked centrilobular necrosis in the liver. The cells in the peripheral lobule were swollen and granular (fig. 5).

Bromsulphalein clearance tests were performed on 2 hospitalized animals suspected of hepatolenticular degeneration (fig. 4,

horses 55-2 and 55-3). Both horses exhibited symptoms of central nervous system lesions and had elevated serum bilirubin levels. Fractional clearance (0.186, 0.195) and half time (3.5, 3.7 minutes) in both animals were within normal limits. Livers were found to be normal at necropsy in each horse. Since intestinal stasis was present in both horses, catarrhal duodenitis could possibly explain the slight hyperbilirubinemia by partial closure of the common bile duct in the absence of a gallbladder. Two other hospitalized animals (54-1 and 54-4) exhibited mild icterus with serum bilirubin levels of 6.8 and 7.2 mg. per 100 cc., respectively. One had a K value of 0.059 with a $t_{1/2}$ of 11.6 minutes and, upon necropsy, exhibited a marked hemosiderosis of the liver. The other showed a K value fo 0.131 with a $t_{1/2}$ of 5.3 minutes and, on necropsy, five days later, extensive centrilobular lipidosis involving one half of each lobule was found. Necrosis was not present (fig. 6).

DISCUSSION

The fractional clearance of BSP during the first 12 minutes is undoubtedly the sum total of many exponential disappearance phenomena. From biliary fistula studies, it appears that the hepatic uptake of BSP accounts for the major part of its elimination from the blood. Extrahepatic uptake and interstitial plasma protein mixing in the horse are poorly understood but, at their greatest, could account for only minor dye losses in the first 12 minutes after injection in control animals. Since hepatic uptake is the greatest factor in BSP clearance, the clearance (c) values in milliliters of plasma per minute should

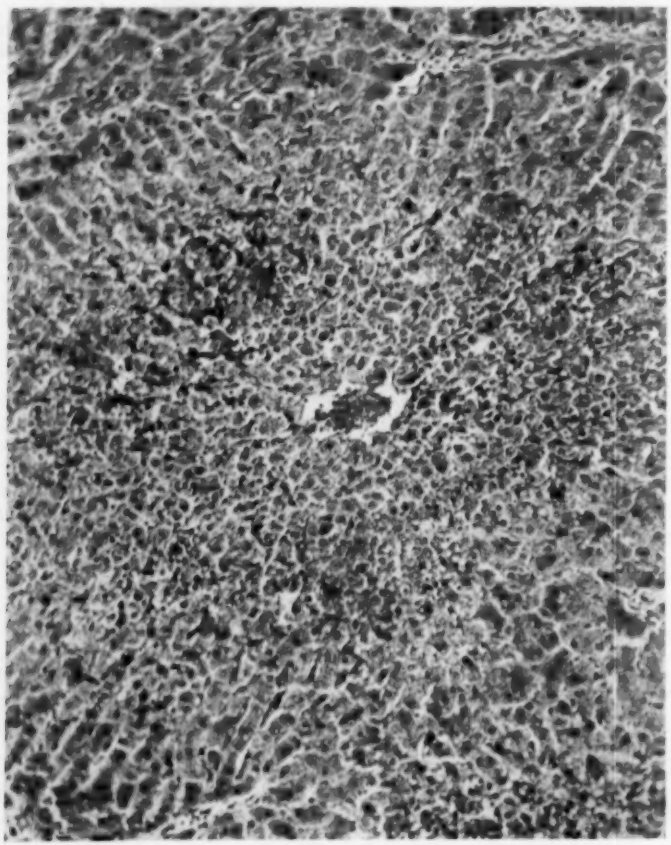

Fig. 5—Section of the liver of horse 55-10 showing centrilobular necrosis following the administration of 250 cc. of CCl_4 per os. x 250.

parallel the hepatic blood flow to some degree. Brauer *et al.*,[9] in studies with BSP distribution in the presence of liver injury in the dog, found that hepatic blood flow may be overestimated to an unpredictable extent as a result of increased extrahepatic uptake. There are many factors which could affect the uptake of a dye from the plasma by the liver. A diffuse necrosis could easily impede hepatic blood flow by generalized swelling of the organ and, in addition, lose enzymatic activity necessary for the acceptance of the dye. Diffuse fatty infiltration in some cases may impede hepatic blood flow and slow the dye clearance without a specific biochemical lesion. Impeding the hepatic arterial blood flow could conceivably affect the dye clearance values more than a relative impediment to portal blood flow.[10] The inability of the liver to excrete the dye could produce a retrograde block which, in turn, would lower plasma clearance.

Fractional clearance between five and 12 minutes after injection is not affected extensively by the enterohepatic circulation of BSP. The fractional clearances within the range of 1.5 mg. of BSP/kg. and 6.3 mg. of BSP/kg. are similar and appear to justify the use of 1 Gm. of dye in horses of differing weights. By this method, plasma volume can be estimated without securing the weight of the horse prior to the test. Following the administration of 1 Gm. of BSP, two plasma samples can be taken at any time interval between five and 12 minutes. From these two concentrations of dye, the K and $t_{1/2}$ values can be calculated and a quantitative assessment of liver function can be made. Calculations can be simplified in veterinary practice by plotting the data on semilog paper and

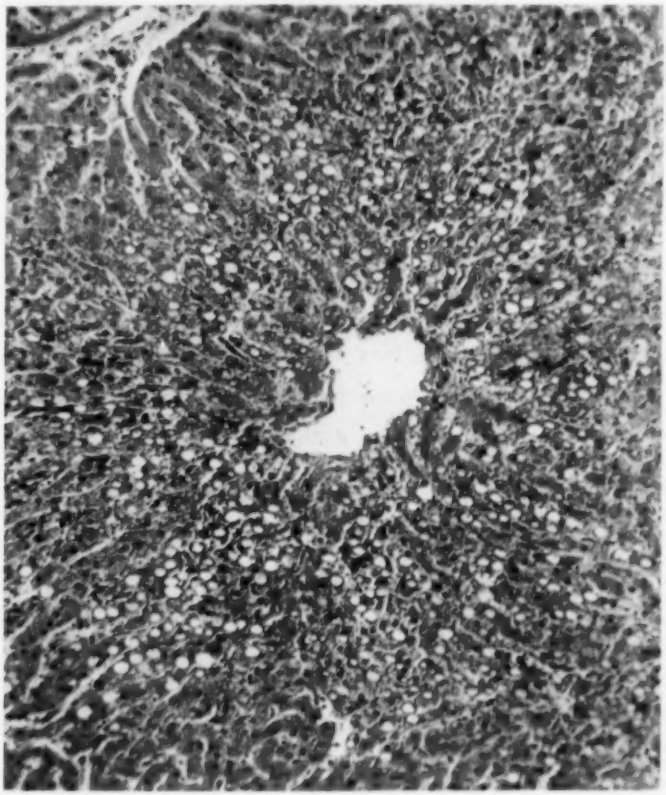

Fig. 6—Section of the liver of horse 54-4 showing extensive lipidosis in the centrilobular area. x 250.

figuring the $t_{1/2}$ directly from this graph paper. The $t_{1/2}$ is that time as measured on the abscissa during which the concentration on the ordinate is halved.

The BSP clearance test has many advantages over the calculation of dye retention. Some disadvantages of the dye retention method are: (1) the animal must be weighed, (2) an exact quantity of dye must be injected, (3) the plasma sample must be taken at one critical time only, and (4) the influences of plasma volume and estimated hepatic blood flow are not assessable. Advantages of the BSP clearance test are: (1) the sampling in the field can be completed in 12 minutes, (2) plasma samples can be taken at any time between five and 12 minutes after injection, and (3) quantitative assessment of liver function can be made in terms of the kinetics of BSP disappearance from the plasma.

SUMMARY

A quantitative liver function test for the horse has been described. The mean fractional clearance (K) for controls between five and 12 minutes after the intravenous injection of bromsulphalein® (BSP) is 0.255 ± 0.05. The mean half-time ($t_{1/2}$) for BSP disappearance is 2.81 ± 0.5 minutes. By extrapolating the semilog plot of dye disappearance to zero time, the plasma volume can be estimated. From the slope (k) of the logarithmic plot, $t_{1/2}$ and K are calculated. The product of the plasma volume (V) and the fractional clearance is the clearance (c) expressed as milliliters of plasma per minute. Variation in the quantity of BSP injected between 1.5 mg./kg. and 6.3 mg./kg. does not alter the fractional clearance. Significant quantities of BSP appeared in the bile 12 minutes after its intravenous injection. Hospitalized horses with confirmed diffuse liver disease exhibited decreased fractional clearances (increased half-times).

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Distemper in Dogs. II. The Response to Vaccination

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IN THE FIRST REPORT of this series, Ott and co-workers[1] demonstrated the prevalence in healthy urban and rural dogs of serum-neutralizing antibody against distemper virus (DV). If antibody reflects the frequency of contact, it appears that the agent is widely disseminated and that contact may occur early in the dog's life. Because of the probability of the young dog's being exposed to the virus, immunization must be accomplished at an early age to minimize canine losses. The purpose of this investigation was to determine factors affecting either or both the serum-neutralizing antibody response and the resistance to challenge with virulent virus of mature and very young dogs and foxes following vaccination.

MATERIALS AND METHODS

Virulent Virus.—Exposure to the Snyder Hill[2*] isolate of DV in foxes and dogs was achieved by intracerebral inoculation. The virus was obtained from a pool prepared from nervous tissue of infected dogs or ferrets. These animals were killed when moribund, and a 10 per cent weight/volume suspension was prepared in nutrient broth (Difco). Antibiotics were added to the diluent in the following concentrations: crystalline penicillin G, 100 units per milliliter, and dihydrostreptomycin sulfate, 100 μg. per milliliter. Ferrets receiving a subcutaneous inoculation of this isolate showed an illness characterized by a prolonged incubation period of 15 to 20 days and intermittent convulsions for 24 to 48 hours until death (fig. 1). Histological examination of the urinary bladde of the ferrets stained by the Pollak method[3] revealed numerous distemper inclusion bodies.

Intracerebral Exposure.—The virus was introduced into the left or right cerebral hemisphere in the following manner. The animals were anesthetized by intravenous inoculation of pentobarbital sodium. Occasionally, ether was employed to produce more profound anesthesia at the time of inoculation. The frontal region was shaved, cleansed with green soap and water, and painted with tincture of merthiolate. A short incision was made through the skin and subcutis and the skull exposed. With a ¼-inch gauge, short-drill bit, a small hole was drilled in the skull. Immediately after removal of the drill bit, a 22-gauge, 1-inch needle, with a syringe attached, was inserted in a vertical direction to the needle hub and 0.75 ml. of virus suspension was slowly inoculated. The skin incision was closed with a single interrupted suture of 32-gauge stainless steel.

Vaccination.—The method of preparing egg-adapted vaccine has been described in detail elsewhere.[4] The preparation was frozen and not lyophilized. At the time of administration, it was removed from the deep freezer (—20 C.), thawed, and each animal given one injection by the subcutaneous route in 2-ml. quantities. A commercial formalin-inactivated vaccine was used in another trial, in which each pup was given three injections of 5 ml. of this preparation at two-week intervals.

Neutralization Tests.—The tests for the detection of distemper antibody were carried out on the chorioallantois of chicken embryos as described previously.[5] The quantity of virus used varied from 100 to 500 units ($i.d._{50}$), as determined by titrations performed concurrently with the tests. Serum samples were secured in the usual manner; from dogs, by the anterior cephalic vein and, from foxes, by cardiac puncture.

Animals.—The ferrets and foxes were obtained from the stock colony of the Fur Animal Disease Station. Litters of young dogs were raised in semi-isolation in the experimental dog units at the Veterinary College. In some instances, unvaccinated pups raised on farms were secured from private sources. These dogs were isolated and kept under observation for at least three weeks prior to use. Only animals in excellent physical condition were included in the experiments.

Criteria of Infection.—The results of intracerebral challenge of foxes and dogs with virulent virus have been arbitrarily divided into three categories: (1) In group 1 were animals with no signs (NS) of distemper infection, neither clinically nor in laboratory findings. (2) In this group were animals with signs (S) of distemper infection on the fourth or fifth day following intracerebral exposure, with temperatures of 104 to 106 F. and a reduced total white blood cell count. Usually the fever persisted for three or four days (in some animals diphasic curves were recorded) and, occasionally, convulsions accompanied by ex-

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This work was supported in part by a grant from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

* The authors are indebted to Dr. V. J. Cabasso for furnishing nervous tissue containing this virus.

cessive frothing at the mouth were observed seven to nine days following inoculation, with improvement on the eighth to the tenth day, and recovery by the twelfth. It would appear that these dogs were partially resistant to challenge. (3) In this group were animals with signs of distemper infection, as previously discussed, but followed by death (D). The temperature reaction and reduction in total count of white blood cells were similar to those of the dogs in group 2, except that hypothermia was registered prior to death. Convulsions were observed in all inoculated animals approximately eight days after exposure. The convulsions were more frequent until the animal became comatose, with death occurring ten to 12 days after inoculation.

EXPERIMENTAL PROCEDURE AND RESULTS

Response of Dogs and Foxes Following Vaccination with Egg-Adapted Distemper Virus.—Ten pups ranging from 6 to 10 weeks of age were vaccinated with living, egg-adapted DV. They were housed in an area which precluded further DV exposure. The *in ovo* neutralization test was used to determine antibody estimates in serums collected immediately prior to vaccination and at regular intervals thereafter (table 1). In some cases, antibody was present before vaccination; probably passively transferred from the dam. The antibody curve was similar to the curve previously reported in that the titer estimates increased for four to six weeks after vaccination and decreased thereafter.[1]

The effect of vaccination on neutralizing antibody and resistance to intracerebral challenge was demonstrated in 4 silver foxes which were given a single inoculation of egg-adapted virus when they were between 3 and 11 months old. They were isolated in an area where there was no opportunity of exposure to DV. The neutralizing antibody curves and the results of intracerebral challenge are described

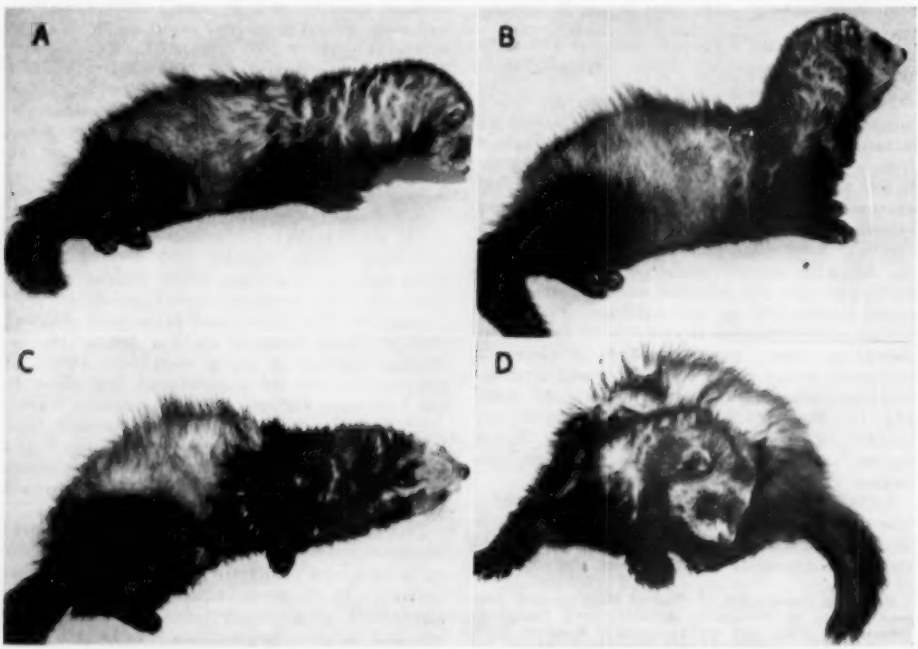

Fig. 1—Distemper, Snyder Hill virus, in ferrets 21 days after inoculation.

A) Marked depression is noted at this time, photophobia but no marked nasal or ocular discharge.

B) Beginning of convulsion—head drawn back, rapid chewing with salivation. Violent screaming may be heard at this time.

C) Height of convulsion. This stage lasts for 30 to 60 seconds.

D) Recovery from convulsion. Staggering gait and other evidence of mental confusion lasting for one to three minutes. Convulsions may be several hours apart during early stage but become more frequent until death.

TABLE 1—The Neutralizing Antibody Response to the Administration of 2 ml. of Egg-Adapted Distemper Virus Vaccine to Pups

Dog No.	Age at time of vaccination (wk.)	Neutralizing antibody estimate of serum*									
		Before vaccination	On postvaccination days								
			7	14	21	28	35	42	56	70	84
186	6	0.3	.....	1.79	.....	2.9	.....	2.9	3.4	3.2	2.79
195	6	0.3	.....	.....	.....	3.4	.....	3.4	3.11	2.69	2.3
519	6	0.3	.....	2.84	.....	2.84	.....	2.9	2.9	2.79	2.3
543	10	0.3	0.3	2.6	2.79	3.5	3.84	3.5	.....	.....	.....
551	7	0	1.79	.....	1.9	.....	2.79	.....	2.6	.....	2.6
571	10	0.3	0.3	2.79	3.11	3.79	4.0	3.69	.....	.....	.....
572	10	0	0.3	1.79	3.79	3.6	3.69	3.3	.....	.....	.....
574	6	0.3	.....	2.84	.....	3.2	.....	3.2	3.11	3.2	2.79
579	6	0.3	.....	2.84	.....	3.0	.....	3.4	3.4	3.9	2.3
580	7	1.3	1.4	.....	3.1	.....	3.3	.....	3.3	.....	2.6
Averages		0.34	0.82	2.49	2.94	3.28	3.52	3.29	3.11	3.16	2.52

* The neutralizing antibody is expressed as the reciprocal of the negative log of the serum dilution (50% neutralizing dose).

(table 2). All animals responded to vaccination with an antibody rise. In foxes 1 and 6, serum-neutralizing antibody ($n.d._{50}$) declined to a low level 7½ months after vaccination; in foxes 33 and 36, it declined at a much slower rate. Twenty-one months following vaccination, neutralizing antibody was still present. The summarized results of challenge (table 2) show that the foxes again developed high neutralizing antibody levels following the administration of the Snyder Hill virus. After challenge, the foxes showed no signs referable to distemper infection, whereas 5 of 5 susceptible dogs and 3 of 5 susceptible foxes inoculated intracerebrally and 2 of 2 ferrets inoculated subcutaneously died of distemper.

Maintenance of the Distemper Antibody by Repeated Kennel Contact.—It has been suggested that DV serum-neutralizing antibody is maintained in the dog by repeated contact with the virus. To study the maintenance of DV-neutralizing antibody in dogs exposed to distemper, the following experiment was carried out.

Two pups from each of three litters were used, and all were vaccinated with a single injection of egg-adapted virus. Those from litter A were vaccinated at 6 weeks of age; from litter B, at 10 weeks; and from litter C, at 7 weeks of age. All were isolated for at least 56 days after vaccination. The litters were then divided and vaccinated pups 1A, 1B, and 1C were isolated. Pups 2A, 2B, and 2C were housed, for a continuous exposure period of 18 days, in an enclosure with dogs exhibiting clinical distemper; 2 susceptible pups were added for control purposes. The vaccinated pups showed no signs of DV infection but the nonvaccinated died with histologically confirmed distemper.

The results of serological studies in relation to antibody level are shown (table 3). As might be expected, the antibody estimates of the 3 vaccinated and isolated dogs (1A, 1B, and 1C) declined, whereas the antibody titer of the 3 vaccinated and exposed dogs (2A, 2B, and 2C) increased slightly. Inasmuch as there was more than a log difference in serum-neutralizing anti-

TABLE 2—The Neutralizing Antibody Response to Vaccination and Results of Challenge with Snyder Hill Virus in Silver Foxes

Fox No.	Neutralizing antibody estimate					Months postvaccination to challenge	Results of challenge
	Before vaccination	1 month postvaccination	7½ months postvaccination	15 months postvaccination	14 to 30 days after challenge		
1	0	2.0	0.5	....	2.3	7½	NS*
6	0	2.5	0.3	....	4.0	7½	NS*
33	0	2.6	1.3	0.8	>3.8	21	NS*
36	0	1.8	1.5	0.7	>3.8	21	NS*
Controls							
5 foxes	....	....	....	....	....	....	3/5†
2 ferrets	....	....	....	....	....	....	2/2†
5 dogs	....	....	....	....	....	....	5/5†

* No signs of distemper virus infection. † Numerator indicates number of animals that died; denominator, the number inoculated.

TABLE 3—The Effect of Contact Exposure on the Serum-Neutralizing Antibody Level of Vaccinated Dogs

Litter No.	Dog No.	Age at time of vaccination (wk.)	Days, postvaccination to challenge	Neutralization titer prior to challenge*	Neutralization titer 3 mo. after challenge*
A	1A	6	Not exposed	2.3	1.3
	2A	6	90	2.79	3.0
B	1B	10	Not exposed	2.3	1.3
	2B	10	112	2.5	2.9
C	1C	7	Not exposed	2.6	1.79
	2C	7	56	2.6	3.3

* Expressed as the reciprocal of the negative log of serum dilution (50% end point).

body titer, it was thought desirable to subject dogs 1A and 2A to intracerebral challenge. Neither dog showed evidence of infection, whereas 2 susceptible controls died. The serological history of these dogs, *i.e.*, before vaccination, kennel exposure in the case of dog 2A, and intracerebral challenge in both dogs, is illustrated (chart 1).

The Neutralizing Antibody Response in Serum from Pups Receiving Killed Tissue Vaccine.—In order to compare the neutralizing antibody response following the administration of killed tissue vaccine with that evoked by egg-adapted distemper virus vaccine, 3 pups, 15 weeks old, were each vaccinated with three 5-cc. doses of a commercial vaccine given at two-week intervals. Eight weeks after the initial dose of vaccine, all dogs were placed in contact with animals with clinical canine distemper, as previously described. None of the vaccinated dogs showed evidence of distemper infection. Subsequent challenge by the intracerebral inoculation of virulent virus, 130 days after vaccination again resulted in no signs of infection. The neutralizing antibody response of these 3 pups is illustrated (table 4).

The Effect of Maternal Antibody on Attempts at Early Immunization of Pups with Egg-Adapted Vaccine.—A bitch, previously vaccinated with egg-adapted DV when 10 weeks old, was hyperimmunized by a single subcutaneous injection of the same material two weeks prior to whelping. The pups were delivered by cesarotomy. Milk samples were taken from the bitch and blood samples from the pups before suckling and at intervals thereafter for 50 days. Equivalent quantities of their serums were pooled and subjected to neutralization tests (table 5).

In this litter, DV-neutralizing antibody was acquired prenatally by placental transmission; however, the greatest quantity received by the pups was in the colostral milk. This observation is in agreement with the findings of Schneider and Szathmary.[6]

Although the serums of the pups reported under the conditions of the neutralization test contained no demonstrable serum-neutralizing antibody at 50 days of age, it has been our experience that antibody may be found in low levels at 70 days of age (table 1). Similarly, mink kits from immunized dams have revealed low levels

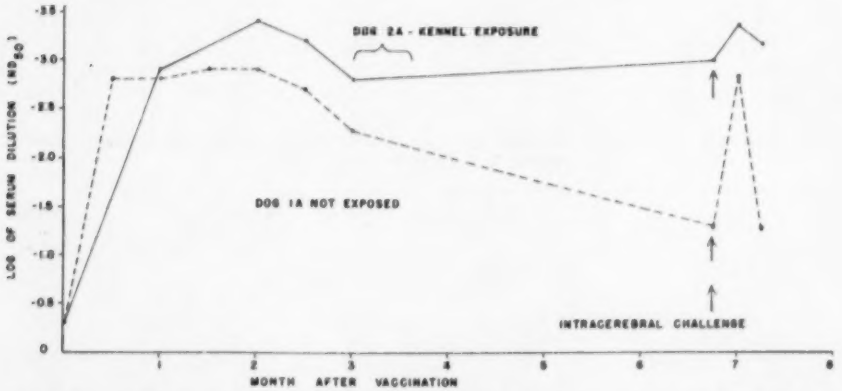

Chart 1—The effect of vaccination, kennel exposure, and intracerebral challenge on the neutralizing antibody titer of dog serum.

TABLE 4—The Neutralizing Antibody Response to the Administration of Formalized Inactivated Vaccine

Dog No.	Age at time of vaccination (wk.)	Neutralizing antibody estimate of serum						
		Before vaccination	On postvaccination days					
			14	28	42†	56	70	130‡	144
SH 1	15	0	0.3*	0.84	0.84	2.3	2.6	3.79	3.79
SH 2	15	0	0	1.2	1.69	1.4	3.4	3.4	3.69
SH 3	15	0	0	0	1.3	1.3	3.4	3.4	3.5

* Expressed as the reciprocal of the negative log of the serum dilution ($n.d._{50}$). † Dogs exposed to animals with clinical cases of distemper. ‡ Intracerebral challenge, 130 days after vaccination.

of serum-neutralizing antibody as long as 73 days following birth.[7]

In order to demonstrate the protective effect of passively conferred maternal antibody against virulent virus, a bitch was hyperimmunized two weeks prior to whelping with a single injection of egg virus and placed in a semi-isolation unit. When the pups were 14 days old, the dam was allowed to mingle freely with distemper-infected dogs. The neutralizing antibody response of a pup of this litter is illustrated (chart 2). The neutralizing antibody curve of the pups raised in complete isolation (table 5) is included in the chart for comparison. The neutralizing antibody of the pup exposed to virulent virus tends to rise rather than decline as does that of the nonexposed group.

Following weaning, the exposed pups were isolated from further contact with DV for 3½ months. At the end of that period, all were challenged intracerebrally with virulent virus. The pups showed no signs of disease, although 2 control dogs challenged at the same time died of distemper.

The possibility of passively transmitted antibody inhibiting the development of active immunity following the administration of egg-adapted virus was investigated using 8 distemper-immune pregnant bitches, 4 of which were hyperimmunized with egg-adapted vaccine. The pups of each litter were vaccinated at varying intervals of 1 to 48 days of age. When possible, 1 or more in each litter were not vaccinated to serve as controls. All were born and raised in isolation units. Their immunity was challenged from 37 to 201 days after vaccination. It is regrettable that no distemper-susceptible bitches were available for this experiment. The results of this trial are reported (table 6). The data would tend to correlate with that previously reported.[8] It was possible to immunize successfully some pups from nonhyperimmunized dams as early as 14 days of age; it was not possible to uniformly protect those from hyperimmunized dams up to, and including, 42 days of age.

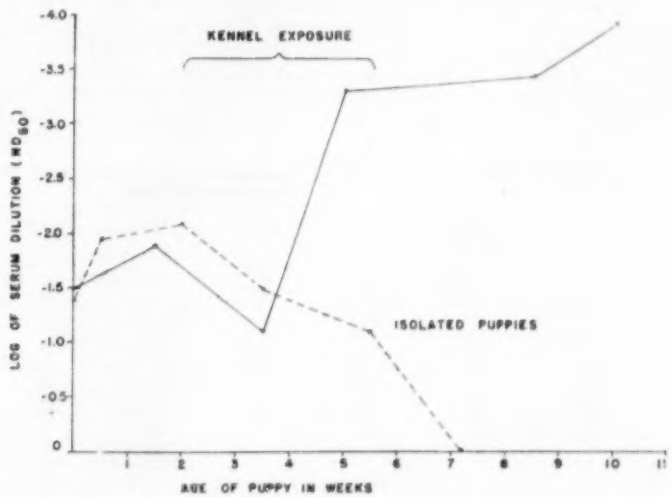

Chart 2—The results of kennel contact with virulent virus on the serum-neutralizing antibody titer of a pup from an immune dam.

DISCUSSION

It should be recognized that distemper in dogs is a common disease which produces a number of mild or asymptomatic attacks for each clinically diagnosed illness. Natural immunization is believed to be an important means of establishing active immunity. A great number of dogs are probably exposed to the virulent agent at a time when the antibody conferred by the dam allows a subclinical infection but prevents frank disease.

TABLE 5—The Results of a Test for Maternally Derived Immunity in the Serum of Pups from a Hyperimmune Bitch

Material tested	At time of birth	4 days	14 days	25 days	38 days	50 days
Pooled serum of puppies	1.4*	1.9	2.1	1.5	1.1	0
Milk from bitch	>3.8	>3.8	....	0.3	....	....

* Expressed as the reciprocal of the negative log of the serum or whole milk dilution ($n.d._{50}$).

The data presented, although limited, would indicate that the antigenic stimulus of formalin-inactivated vaccine was sufficient in this case to provide resistance. Inasmuch as the egg-adapted DV multiplies in the tissues of the host,[9] presumably a more extensive immunological stimulus may result than that provided by a definite quantity of killed vaccine. Furthermore, it is safe to say that the antigenic characteristics of the virus are more likely to be maintained in a live agent than in a chemically or physically killed vaccine.

To be a successful antigen, egg-adapted distemper vaccine must be living at the time of administration. In a recent experiment, 60 ferrets received egg-adapted vaccine which was believed to be living when injected. All 60 animals succumbed to later challenge,[9] and studies revealed that the egg material was dead at the time of vaccination.

Not infrequently, vials of commercial egg-adapted vaccine are submitted for viability testing. In many instances, the initial growth on the chorioallantoic membrane consists of a limited number of opaque pocks which are representative of virus. Edema is rarely observed. Occasionally, lesions are not found following initial *in ovo* incubation, and further passages are necessary to demonstrate virus. To test for virus which might be present, although visually undetectable on the chorioallantoic membrane, this vaccine is occasionally given to young dogs. These animals may show no demonstrable neutralizing antibody response and later succumb to challenge.

Vaccination should be accomplished when the passive antibody conferred by the dam is at a level which will not interfere with active immunization. Our previous studies showed the influence of the immune status of the dam.[8] In this trial, the offspring of vaccinated ferrets were refractive to immunization until the thirty-sixth or the forty-seventh day following birth, whereas the young from susceptible ferret dams were successfully immunized when 8 days old. An attempt was made to repeat this work in young dogs. Although pups of some immune dams could be immunized when vaccinated at 14 days of age, those from hyperimmunized dams could not be uniformly protected by vaccination at 42 days of age.

It might be hypothesized that pups from distemper-susceptible dams could be successfully immunized with egg-adapted virus when a few days old; however, the

TABLE 6—The Results of Early Vaccination and Challenge of Pups from Immune and Hyperimmune Dams

From immune dams				From hyperimmune dams			
Dog No.	Age at time of vaccination (da.)	Days after vaccination to challenge	Results of challenge	Dog No.	Age at time of vaccination (da.)	Days after vaccination to challenge	Results of challenge
189	6	176	S	55-1-1	1	53	D
197	14	176	NS	55-2-1	12	93	D
581	14	176	NS	55-5-1	21	78	S
544	14	176	NS	55-6-1	21	58	D
549	33	149	S	55-5-2	28	71	S
599	40	201	NS	55-6-3	28	51	D
582	48	98	NS	55-5-3	35	64	D
5 controls	....	.....	D	55-6-4	35	44	D
				55-6-5	35	44	D
				55-5-4	42	57	S
				55-6-6	42	37	NS

S = signs of distemper virus infection; NS = no signs of distemper virus infection; D = died.

practitioner should be warned that only under exceptional circumstances would such bitches be encountered.

Slanetz [10] has shown that virulent distemper virus may overcome the maternal immunity of the suckling pup with the production of active immunity. The use of a vaccine which contains a mixed population of virulent and modified virus particles may also be effective at an early age. Such vaccines are under investigation.

The effect of repeated antigenic stimuli with egg-adapted virus at intervals should be studied. Such a procedure might overwhelm high levels of serum-neutralizing antibody passively conferred by the dam.

In any attempt to translate the results of these laboratory experiments in terms of the protection afforded dogs against field exposure, it should be borne in mind that the amount of virus introduced by natural exposure is usually less than that employed by the laboratory challenge tests.

It has been shown that the serum-neutralizing antibody estimates following the administration of a modified live virus distemper vaccine reach their ceiling at approximately four to six weeks postvaccination and, thereafter, decline slowly for 21 months or more. Immunity challenge of these vaccinated animals, when their serum antibody level approached 0, revealed that they were refractory to intracerebral inoculation with virulent virus. As compared with susceptible animals, the differences in neutralizing antibody may be nondetectable. Tests for antibody vary widely in their sensitivity, and negative results can not be interpreted to mean that no antibody is present; they merely indicate that antibody is not detectable with the particular test used.

In a previous report,[1] it was suggested that repeated exposure to virulent virus would maintain the antibody at a high level in urban populations. A laboratory study has validated this. Although it is hazardous to speculate, it might be suggested that vaccination of pups with egg-adapted modified living virus should be accomplished at 6 to 8 weeks of age. It is possible that in some instances at that age antibodies passively transferred from the dam may interfere with this vaccine. Repetition of the vaccination two weeks later would tend to minimize this interfering effect.

Two final reservations should be made regarding immunization against distemper. The first is that we can never expect complete success from any preventive measure. Dogs differ greatly in their susceptibility to infection and in the readiness with which they respond with antibody production. It is as foolish to condemn a method for a few failures to protect as to proclaim it successful because of a few instances of apparent protection.

CONCLUSIONS

1) Egg-adapted distemper virus (DV) stimulates a rise in serum-neutralizing antibody which reaches its ceiling in approximately four to six weeks and, thereafter, declines.

2) High levels of neutralizing antibody were maintained in a group of laboratory pups by repeated pen contact with animals with distemper.

3) Suckling pups which have received maternal antibodies prenatally by placental transmission and postnatally in the colostrum may respond to virulent exposure by development of active immunity without manifestations of frank disease.

4) The immune status of the dam affects the immunizability of the young pup. High levels of passively transmitted maternal immunity may interfere with early vaccination using a single dose of egg-adapted virus.

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A New Feline Virus Isolated in Tissue Culture

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DURING a project initially undertaken to grow in tissue culture the virus of panleukopenia (feline enteritis), an unrelated agent which produced intense cytopathogenic changes in kitten kidney epithelial cells was encountered. The present paper describes (1) attempts to characterize this latter virus and (2) the results of a limited serological survey carried out to determine its distribution amongst apparently healthy cats.

MATERIALS AND METHODS

Tissue Culture Procedure.—Monolayer sheets of kitten renal epithelial cells were prepared by the trypsinization technique of Youngner.[1] Donor animals, 4 to 10 weeks of age, were exsanguinated under nembutal® anesthesia and the kidneys were removed aseptically. After rejecting the medullary areas, the minced cortices were repeatedly extracted at 37 C. with fresh volumes of 0.25 per cent trypsin (Difco, 1:250) in Hanks' balanced salt solution (BSS) until cellular exhaustion was indicated by floating tissue debris. The combined cell suspensions were washed in three successive changes of fresh BSS and finally nutrient medium was added to form an 0.75 per cent suspension in terms of packed cell volume. This stock suspension was distributed among 6- by ⅝-inch test tubes (1 ml.) or 4-oz. flat prescription bottles (8 ml.). During subsequent stationary incubation at 37 C., a single medium change, made on the fifth day, sufficed to produce by the eighth to ninth day semiconfluent cell sheets suitable for virus inoculation. The nutrient medium used throughout consisted of Hanks' BSS (80.0%), heat-inactivated normal horse serum (10.0%), 5 per cent lactalbumin hydrolysate (10.0%), and 100 units per milliliter each of penicillin and streptomycin. The final pH was within the range of 7.6 to 7.8. For detailed histological study, monolayer cultures formed either in Carrel flasks or directly on cover slips were fixed with absolute methanol and subsequently stained by Giemsa's rapid method.

Survival cultures involving tissue fragments suspended in nutrient medium were usually prepared in tightly stoppered 50-ml. conical flasks. The selected tissue was finely minced, thoroughly washed in BSS, and approximately 30 pieces, 1 mm. square, were transferred to flasks containing 3 ml. of nutrient medium. Following virus inoculation, all flasks were incubated at 37 C., some being rocked continuously at a speed of 40 r.p.m. Agitation was not, however, consistently employed. It was customary to replace completely each fluid phase after the first six hours of incubation, thereafter only the pH being readjusted to 7.2 to 7.4 when necessary. Passage was accomplished by grinding the contents of each flask in a TenBroeck homogenizer, centrifuging lightly, and transferring the supernatant to freshly prepared cultures.

Both in suspended cell and in monolayer cultures, the inoculum volume was such that an immediate dilution of 1:10 was effected. In calculating the virus dilution at each medium replacement, no allowance was made for the small volume of fluid unavoidably retained by clusters of tissue fragments or of cell sheets.

Assays for Virus.—Virus-containing material was diluted in tenfold steps with nutrient medium, a fresh pipette being used for each dilution. A number of preformed, kitten kidney, monolayer culture tubes (8-day growth) sufficient for the titration series were drained of their medium and every virus dilution inoculated into at least three tubes, each receiving a volume of 0.8 ml. After three days' incubation at 37 C., all tubes were examined and the presence or absence of cytopathogenic changes were recorded. Virus infectivity was calculated by the Reed and Muench method[2] and expressed as the 50 per cent tissue culture end point (TC_{50}).

Neutralization Tests.—All serums were diluted 1:5 in physiological saline and inactivated at 56 C. for 30 minutes. Serial twofold dilutions were then prepared in tissue culture medium and to each was added an equal volume of virus suspension diluted to contain between 200 and 2,000 TC_{50}. The mixtures were held for one hour both at 37 C. and at 4 C.; then 0.8 ml. of every dilution was inoculated into each of four preformed, kitten kidney, monolayer cultures. After three days' incubation at 37 C., the highest serum dilution (prior to mixing with virus) which completely suppressed the cytopathogenic effect in two or more tubes was accepted as the antibody titer. For screening purposes, all serums were used in final dilutions of 1:25, 1:50, and 1:100, full titrations being performed when an end point was not reached by this dilution range.

Immune Serum Production.—Blood samples were taken routinely before exposure and thereafter at weekly intervals from all kittens and adult cats employed in transmission experiments with the newly isolated virus. These serums were kept at −25 C. in the unheated state. Three in-

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The author is indebted to Mr. A. Bryant, Animal Research Station, Wallaceville, New Zealand, for the photographs reproduced in this article, and to Miss Jennifer Maseyk for technical assistance.

travenous injections (each of 5 ml.), of infected kidney monolayer culture fluid given to rabbits at weekly intervals sufficed to produce a hyperimmune serum. An animal receiving a similar immunizing schedule with noninfected fluid provided a serum control upon possible nonspecific interference by antibodies formed against normal components of the culture medium. Subsequent neutralization tests showed that this control serum failed to prevent cytopathogenic changes produced by the kidney cell-degenerating agent in the lowest dilution tested (1:25), whereas the virus hyperimmune serum possessed an inhibition titer of between 1:800 and 1:1,600. A panleukopenia hyperimmune serum, produced in an adult cat by repeated intraperitoneal injections of virus-infected tissue, was also employed. In the latter instance, two doses of commercial panleukopenia vaccine* were administered prior to the living virus.

RESULTS

Primary Isolation of the Agent.—After treatment with penicillin and streptomycin, a 10 per cent suspension of the spleen, lymph nodes, and kidneys of a cat moribund from a laboratory-induced panleukopenia infection was inoculated into ten tubes containing preformed sheets of kitten kidney epithelial cells. The medium was renewed on the first day of incubation and both cellular and fluid phases harvested after a further five days at 37 C. No cytopathogenic changes were apparent. The cultures were twice frozen and thawed to disrupt the cells, combined, and after slow speed centrifugation, the supernatant fluid added to fresh monolayer cultures. Here again, no cytopathogenic changes were noticed throughout a further incubation period of six days. During the third passage, however, some minor degenerative changes, which first appeared on the third day, became more pronounced until, by the fifth day, the majority of cells were rounded and had become detached from the glass surface. These cultures provided the infectious material from which stemmed a series of passages in kitten kidney monolayers, each inoculum consisting of the preceding culture harvested at a time when maximal degeneration occurred. By the sixth transfer, when the original inoculum had undergone a theoretical dilution of 10^{-12}, the culture possessed a TC_{50} of $10^{-6.8}$. Thus, ample evidence was provided as to the transmissible nature of the cell-degenerating agent. As the number of passages increased, there was an acceleration in the time at which cell death was apparent. The changes initially observed on the third to fourth day were soon occurring within 18 hours and to avoid possible cytotoxic effects due to an excess of preformed virus, inoculums for further passages were diluted at least a thousandfold.

Once the virus had been satisfactorily established in tissue culture, an attempt was made to ascertain its origin. The possible contamination of monolayer cells by an extraneous virus present either in the medium or in the donor kidney tissue itself could readily be excluded since at least ten uninoculated cultures were routinely included with each virus passage. No degenerative changes were found in the contents of any of these control tubes. A second passage line was, therefore, initiated with the original panleukopenia virus-infected cat tissue and, after six transfers, a cytopathogenic agent was again recovered. That the virus of panleukopenia was not, however, responsible for the observed degeneration, was suggested by the inability of two other local isolates of the former virus to produce any change in kitten monolayer cultures even after eight consecutive passages in these cells and also by neutralization tests reported elsewhere.

Throughout this paper it is proposed to designate the present cytopathogenic agent as the "kidney cell-degenerating virus," abbreviated as "KCD virus." The term refers to its marked effect upon cultures of feline kidney and is not intended to imply that either *in vivo* predilection for this tissue is exhibited or that the observed effects on cat tissue *in vitro* are restricted to renal epithelial cells.

Growth Characteristics.—Using both stained and unstained cover slip preparations, a study was made of the early phases of KCD virus growth in kitten kidney monolayer cultures. Following the inoculation of approximately 100 TC_{50} virus, incipient cytopathogenesis was first recognized in living, unstained cultures by the cytoplasm of several cells becoming highly refractile within ten hours of exposure to virus. This was accompanied by a considerable increase in the size of individual cells. Three to five hours later, these changes extended to adjacent areas of the monolayer sheet, the cellular components eventually con-

* Tasman Vaccine Laboratory, Ltd.

tracting to oval shapes with the residual cytoplasm maintaining contiguity with the neighboring uninfected cells through fibrillar processes (fig. 1). Upon staining by Giemsa's method, the cells involved in this process were found to possess pyknotic nuclei and intensely basophilic cytoplasmic strands. At a similar stage of infection, macroscopic examination of tube cultures, after staining with phloxine and methylene blue, showed these areas as clear plaques upon a mauve ground (fig. 2). By the eighteenth hour of incubation, infected cells were seen as rounded, clumped masses which soon detached from the glass wall and floated freely in a fluid phase, the pH of which was 7.6 to 7.8 (fig. 3). As distinct from the behavior of a number of animal viruses in tissue culture, syncytial structures or giant cells were not observed during growth of the KCD virus. No inclusions could be demonstrated at any stage of virus multiplication *in vitro*, nor could elementary bodies be seen following the Macchiavello staining of cultures possessing high virus titers (TC_{50} $10^{-7.2}$).

The rate of release of virus from infected kidney epithelial cells was studied in the following manner. A large number of tubes containing preformed monolayer sheets were exposed to approximately 100 TC_{50} of KCD virus at 37 C. for 30 minutes. The fluid phase was then completely replaced with fresh culture medium and all tubes returned to the incubator. At intervals of three hours thereafter, fluids from at least four cultures were removed, pooled, and stored at —25 C. In a similar manner, infections initiated with a virus inoculum of 1,000 TC_{50} were studied. At the conclusion of both experiments, all harvests were titrated for infectivity in trypsinized kidney cell cultures (graph 1).

After an initial period of four to six hours during which time little virus could be detected, infectivity rose rapidly until, by the twenty-first hour, maximal titers were present for the 1,000 TC_{50} inoculum series. Where the initial exposure was to 100 TC_{50}, virus release was somewhat slower; in both cases, however, comparable yields of infective virus were eventually produced. Further experiments using virus inoculums in the range of 1 to 5 TC_{50} showed stable cytopathogenic end points to be reached after 60 hours at 37 C. It is for this reason that an incubation period of three days has been allocated for all in-

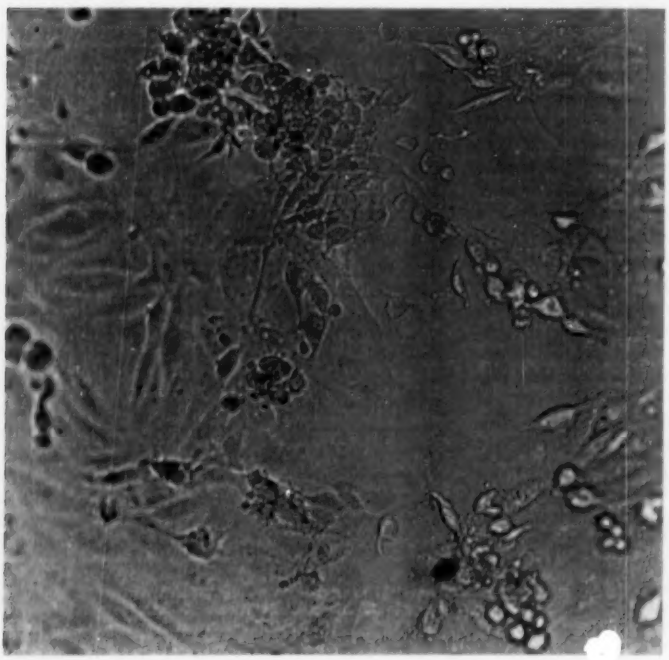
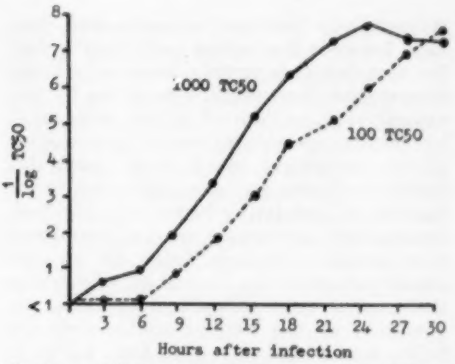


Fig. 1—Initial stages of KCD virus action on kitten kidney epithelial cells. Individual cells becoming highly refractile and rounded in shape. Normal tissue sheet at lower left. Unstained culture. x 150.



Graph 1—Effect of inoculum size upon growth curves of KCD virus in kitten kidney monolayer cultures.

fectivity titrations and neutralization tests with the KCD virus. In the latter procedure, this time limit was of considerable importance for virus "breakthrough" of otherwise neutral mixtures occasionally occurred with further incubation, this being a comparatively common feature in tissue culture-neutralization tests.[3]

The ability of different kitten tissues to support virus growth *in vitro* was determined, using in each case as inoculum the virus produced from the eighth passage in kidney monolayer culture. After an initial contact period of six hours at 37 C. between virus and tissue fragments, the medium was completely replaced. Apart from correcting small pH reductions 18 hours later no further adjustments were made until the cultures were harvested on the third day f llowing preparation. In this manner, five successive passages were made in separate cultures of spleen, kidney, adrenals, lung, brain, liver, mesenteric lymph nodes, and shavings of the submucosal layers of the ileum. In each case, cell death with subsequent autolysis of tissue fragments was shown by elevated pH values (7.6 to 7.9) which persisted despite continued incubation of the cultures. When infectivity titrations in kidney monolayers were undertaken, the ground whole cultures gave TC_{50} end points of kidney of $10^{-6.2}$; liver, $10^{-4.0}$; lymph node, $10^{-3.8}$; adrenals, $10^{-5.8}$; lung, $10^{-5.4}$; brain, $10^{-4.2}$; ileum, $10^{-5.2}$; and of spleen

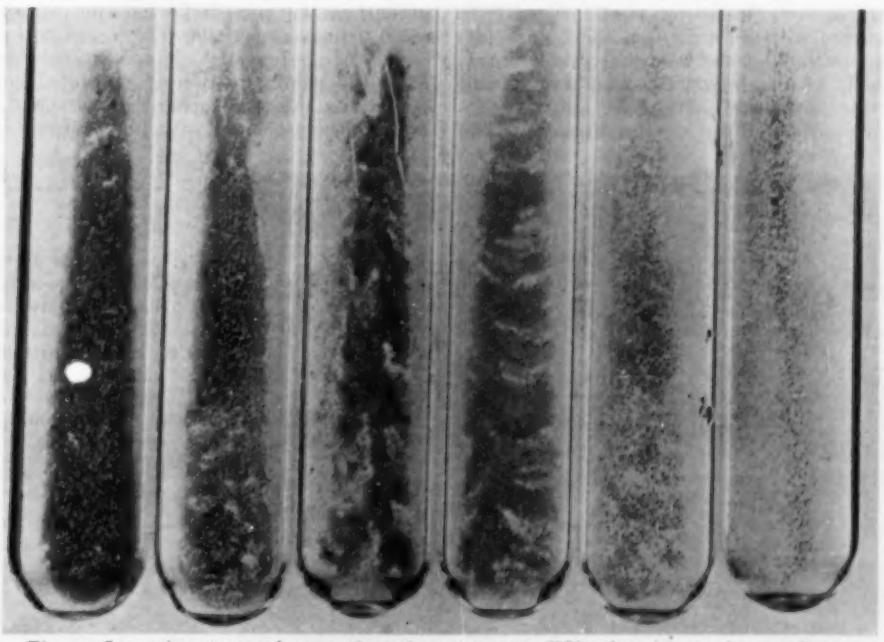


Fig. 2—Successive stages of cytopathogenic action with KCD virus in monolayer cultures. Normal kitten kidney epithelial cells (left), complete degeneration (right). Phloxine methylene blue stain. x 1.4.

$10^{-3.8}$. It is thus apparent that under *in vitro* conditions, growth of the KCD virus could be supported by a wide variety of cat tissues. While the method by which the suspended cultures were prepared does not permit accurate comparison between the yield of virus from each type of tissue, low titers appeared to be consistently produced by lymph node and by spleen fragments.

Of several kitten tissues incubated in the form of conventional plasma clot cultures, only ovarian tissue produced luxuriant fibroblast outgrowths. These preformed cells underwent degeneration when exposed to KCD virus in dilutions up to $10^{-5.0}$, as compared with $10^{-7.0}$ when other portions of the same virus dilutions were inoculated into kidney cell monolayer cultures.

Host Range.—Despite the marked pantropism of KCD virus for feline tissues *in vitro*, no obvious clinical signs were produced in kittens following its administration by a number of routes. Only young animals 6 to 10 weeks of age were employed, each being bled before inoculation and again 21 days later. Daily rectal temperatures were also recorded. The sixth monolayer culture passage (TC_{50} $10^{-6.8}$) was inoculated into groups of 2 or 3 kittens by each of the following routes, intranasal, 1 ml.; intraperitoneal, 4 ml.; and intravenous, 3 ml. Of these, only the kittens inoculated by the intravenous route showed some transient anorexia and diarrhea between the eighth and twelfth day. No significant departure from the normal temperature fluctuation was shown by any animal. In the case of kittens inoculated by the intraperitoneal route, examination of the peripheral blood every third day failed to disclose any gross variation in the number of circulating leukocytes. Neutralization tests performed on the preinoculation serum specimens from all animals showed titers of less than 1:25, while those undertaken upon serum removed after inoculation by the various routes gave the following end points: intranasal, 1:200, 1:400, and 1:200; intravenous, 1:800 and 1:400; and intraperitoneal, 1:800, 1:3,200, and 1:3,200.

Direct intracerebral inoculation of the KCD virus failed to produce signs in either suckling or in weaned mice despite four "blind" transfers by this route. No virus could be demonstrated when brains, from animals of both age groups, were removed three, six, or 12 days after infection and subinoculated into kitten kidney monolayer cultures. Similar results attended a search for residual virus in the lung tissue of half-grown mice following the intranasal instillation of virus. There was no evidence of pulmonary consolidation even after the administration of undiluted culture virus, thus indicating the absence of a toxic effect asociated with high concentra-

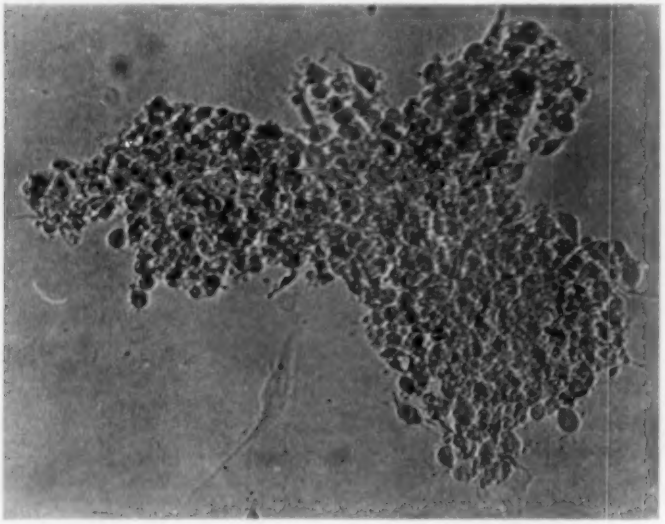


Fig. 3—Complete degeneration of culture shown in figure 1. Twenty-four hours after infection with KCD virus. Unstained culture. x 150.

tions of the KCD virus. The inability of murine tissue to support viral growth was later confirmed by *in vitro* studies. Here, brain and viscera from both embryonic and suckling mice, following initial exposure to the KCD virus as suspended tissue fragments, proved to be free of this agent after a subsequent incubation period of four days at 37 C. in culture medium.

The seventh monolayer passage of KCD virus was injected intracerebrally into 1-day-old guinea pigs without adverse effect. Similarly, half-grown rabbits showed no clinical response to an intravenous inoculation of 5 ml. of culture fluid highly infectious for kitten kidney cells.

All attempts to infect chicken embryos were unsuccessful. These included blind passage by the chorioallantoic membrane, the amniotic, the yolk sac, and the allantoic routes. However, rapid alternate passages on the chorioallantoic membrane and then in kitten kidney monolayer cultures showed that infectivity for the latter tissue could at least be maintained over five transfers—this contrasting sharply with the complete disappearance of KCD virus from the membrane during a single prolonged incubation (5 days) *in ovo*. However, it is highly probable that under the conditions of alternate host transfer virtually no growth occurred in the chicken embryo membrane because continued propagation of the KCD virus depended entirely upon residual virus being fostered by each kitten tissue culture passage. In monolayer cultures of chicken embryo liver cells, as in plasma clot preparations of heart fibroblasts, no cytopathogenic changes followed exposure to the virus. The supernatant fluids removed from such cultures after five days' contact did not induce degeneration in kitten kidney monolayers. Similar results attended several attempts to grow the KCD virus in cultures composed of trypsinized dog kidney tissue and of sheep embryo cutaneous muscle.

Filterability and Resistance to Physical Agents.—Monolayer culture fluids of the KCD virus retained infectivity after passage through serum broth-treated Seitz EK and Berkefeld W filters. Tested at a 1:10 dilution, other portions of the same unfiltered virus suspension proved to be noninfective after minimal exposure times at 56 C. for eight minutes and at 60 C. for four minutes. Virus could be recovered from refrigerated (4 C.) infective tissue culture fluids for at least ten days, while the TC_{50} of KCD virus suspensions after storage at —23 C. for three months was two logs of dilution lower than that of the fresh material. Following precipitation with protamine sulfate in the proportion of 5 mg. per milliliter of culture fluid (Warren *et al.*[4]), virus was recovered to almost full titer in the supernatant fluid. Exposure of 1 volume of KCD virus fluid to 2 volumes of anesthetic ether at 4 C. for different periods of time resulted in the following reductions in infectivity: control, $10^{-7.2}$; two hours, $10^{-6.6}$; four hours, 10–$10^{-6.0}$; and 18 hours, $10^{-5.4}$.

Hemagglutination.—Red blood cell suspensions from different animal species were used in an attempt to demonstrate viral hemagglutination. Tests were conducted in duplicate at 4 C. and at 37 C., using as diluents for both virus and erythrocytes 0.15 M sodium chloride buffered with 0.02 M phosphate at the four pH levels of 6.5, 7.0, 7.5, and 8.0. The KCD virus suspension, being a pool of five kidney monolayer cultures at the eleventh passage level, possessed a TC_{50} of $10^{-7.2}$. Under these conditions, no specific hemagglutination occurred with group O human, guinea pig, sheep, rabbit, mouse, or 1-day-old chicken cells. Unlike work on the hemagglutinin of meningopneumonitis virus by Hilleman *et al.*,[5] the use of a citrate diluent failed to disclose a calcium or magnesium ion masking effect.

Neutralization Tests.—In addition to the serological tests reported earlier, hyperimmune serums prepared against the viruses of panleukopenia and of lymphocytic choriomeningitis (Jermain strain) were employed to exclude their possible relationship to the KCD virus. At the lowest dilution tested (1:25), neither serum inhibited the cytopathogenic effect produced by the normal test dose (100 to 1,000 TC_{50}) of KCD virus. Two further panleukopenia immune serums obtained from cats convalescing from a laboratory-induced infection likewise possessed no protective effect *in vitro*, although 21 days after the intraperitoneal inoculation of 2 ml. of KCD virus-infected culture fluid, the neutralizing titers of these animals rose to 1:800 and 1:3,200, respectively. A limited number of serums derived from apparently normal

cats of all ages were screened to determine their antibody content against the KCD virus.

TABLE 1—Distribution of KCD Virus-Neutralizing Antibodies in Serums of Apparently Healthy Cats

Approximate age (weeks)	No. of animals	Serum-neutralization titer					
		<25*	25	50	100	200	400
4 to 12	15	12	2	1	—	—	—
12 to 18	18	4	8	4	1	1	—
>18	10	2	—	3	2	2	1

* Reciprocal of serum dilution preventing the cytopathogenic effect of 100 to 1,000 TC_{50} doses of KCD virus.

The results (table 1) suggest that the degree of association with this agent sufficient to elicit a detectable serological response is more common in adult cats than in recently weaned kittens.

DISCUSSION

Among the feline diseases of possible viral origin, only those agents responsible for one form of pneumonitis (Baker's virus) and for panleukopenia have been adequately characterized. It is, therefore, with these two viruses that in the first instance the recently isolated kidney cell-degenerating agent must be compared. Baker's virus[6] possesses many of the properties characteristic of the psittacosis-lymphogranuloma group. Thus, it may readily be communicated to mice by both intranasal and intracerebral routes, and to the yolk sac of the developing chicken embryo. In all three localities, subsequent growth results in the formation of well-defined elementary bodies. The KCD virus, on the other hand, has so far resisted attempts to infect any animal species other than the cat. Indeed, even in this host, the virus is of such low virulence that in the living animal the detection of neutralizing antibodies subsequent to inoculation forms the only basis whereby infection may be recognized. This is in distinct contrast to the pantropic tendencies exhibited by the KCD virus for a variety of kitten tissues *in vitro*. However, despite their possession of high virus titers, these cultures, when stained either by Giemsa or Macchiavello techniques, have not shown the presence of recognizable elementary bodies.

The KCD virus may be considered distinct from that responsible for panleukopenia on the grounds that in tissue culture antibodies to the latter have been found incapable of neutralizing the intense cytopathogenic action of the former virus. Furthermore, kittens immunized against panleukopenia and at the time possessing no neutralizing antibodies for the KCD virus developed the latter to a high titer following a subsequent injection of infected tissue culture fluid.

The uncertain origin, together with an inability to produce any overt clinical response when inoculated into supposedly susceptible animals, contributes toward placing the KCD virus in a position formerly held by the Coxsackie viruses, *viz.*, a filterable agent in search of a disease. Indeed, the isolation of viruses from the tissues[7,8] and excreta[9-11] of apparently healthy human and lower animals makes for increasing awareness not only of the direct role as infectious agents of these aptly termed "orphan viruses"[12] but also of their possible synergistic or antagonistic influence upon other viral diseases. The KCD virus appears to bear an ecological resemblance to the agents responsible for spontaneous degeneration of cultures of monkey kidney epithelial cells[13-15] and of human adenoidal tissue[7] for, in each instance, growth is restricted to tissues of the animal species from which the virus was initially derived. Furthermore, despite the inability to demonstrate any apparent illness, many individuals have been shown to possess serological evidence of past experience with the appropriate orphan virus. In the present instance, a limited number of neutralization tests performed with the KCD virus upon serums from cats of different ages suggest that infection is on the whole acquired during adult life. However, in the absence of clinical symptoms, the role played by this virus as a disease-producing entity must await the results of further study.

SUMMARY

The isolation, growth characteristics, and host range of a virus responsible for intense cytopathogenic changes in kitten kidney tissue cultures have been described. Both the low virulence for cats and restriction of *in vitro* growth to tissues from this animal species suggest that the agent

might best be regarded as an "orphan virus" of cats.

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Neutralization and Titration of Newcastle Disease Virus in Chicken Embryo Tissue Cultures

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THE PRESENT STANDARD METHODS of titrating most viruses, including Newcastle disease virus (NDV), involve the use of test animals or embryonating eggs, and the results are expressed in $l.d._{50}$ or $i.d._{50}$. Similarly, the resulting change in $l.d._{50}$ or $i.d._{50}$ when the serum of an immunized animal is mixed with a given virus (neutralization index) is used as a measure of the degree of immunity of the animal whose serum is tested. These methods require the use and maintenance of large stocks of laboratory animals or embryonating eggs, both expensive and space-consuming.

However, in the last few years, new techniques of preparing tissue cultures and growing viruses in them have been developed, and these have made possible, in many cases, the performance of virus titrations and neutralization tests in the test tube. Newcastle disease virus has been shown to be no exception and the cultivation of this agent or its titration have been described in tissue cultures of HeLa cancer cells, chicken embryo lung, chicken embryo liver,[20] Ehrlich ascites carcinoma,[9,17] chicken embryo fibroblasts,[1,14] adult chicken and rabbit tissues,[12] skeletal muscle tissue of chicken embryos,[8] chicken embryo heart muscle,[15] chorioallantoic membrane,[11] monkey kidney,[3] human embryonic lung,[4] and in this laboratory, in cultures of human amnion.[22]

This paper describes the use of tissue cultures of chicken embryo fibroblasts for the titration of NDV and for the determination of the neutralization index of serums of animals immunized against this virus.

MATERIALS AND METHODS

Cleaning of Glassware.—All glassware used for the growth of tissue cultures was heated in an oven to 500 C. for one hour. The necessity of maintaining separate stocks of glassware for these purposes or the use of special detergents or acids in cleaning was thus obviated.

Virus Stock.—The strain of NDV used in these experiments was isolated in Israel and is highly pathogenic for chickens.[13] This strain was designated as HP. Infected allantoic fluid from the fifty-sixth to sixty-fourth passages of the virus in embryonating eggs was used in all experiments described in this report.

Mediums.—Four different mediums were used in the course of the experiment. Growth medium M1[16] consisted of Hank's balanced salt solution containing 1.0 mg./ml. of glucose, 97.0 ml.; lactalbumin hydrolysate, 0.5 Gm.; and calf serum, 2.0 ml. Growth medium M4 consisted of Hank's balanced salt solution containing 0.1 mg./ml. of glucose and 2.4 mg./ml. of sodium acetate, 97.0 ml.; lactalbumin hydrolysate, 0.5 Gm.; and calf serum, 2.0 ml.

Maintenance medium M3 consisted of Hank's balanced salt solution containing 0.1 mg./ml. of glucose and 2.4 mg./ml. of sodium acetate, 100.0 ml., and lactalbumin hydrolysate, 0.5 Gm. Maintenance medium M2 consisted of Hank's balanced salt solution containing 0.1 mg./ml. of glucose, 100.0 ml., and lactalbumin hydrolysate, 0.5 Gm.

All mediums contained 100 units/ml. of penicillin and 50 μg./ml. of streptomycin. Mediums were sterilized by filtration through an EK sterilizing Seitz filter.

Mediums M1 and M4 were used for the growth of chicken embryo tissue cultures. In order to maintain the cells in a viable condition without allowing multiplication, the M1 or M4 mediums were replaced with mediums M2 or M3—this was usually done after 24 hours of incubation in the growth medium. The calf serum used was derived from calves 6 to 12 months old.

Immune Serums.—Immune serums were obtained from chickens, 6 weeks old, which survived an infection with strain HP, and 14 days after an intramuscular challenge with the same virus. The hemagglutination-inhibition (HI) titers of these serums ranged from 160 to 320 and their neutralization index, as determined in embryonating eggs, was 400,000.

Preparation of Tissue Cultures.—Cell suspensions were prepared in a manner similar to that described by Dulbecco.[6] Nine- or 10-day embryos were removed from the eggs aseptically and, after their heads and legs were cut off, they were pressed through a 24-mesh, stainless steel, wire grid in the bottom of a 50-ml. syringe into a vessel containing Earle's balanced salt solution. After allowing the pulp to settle for 10 minutes, the supernatant was sucked off and replaced with 0.2 per cent trypsin in Earle's solution. This suspension was incubated in a water bath at 37 C. for 20 minutes, dispersed with the aid of an auto-

From the Israeli Institute for Biological Research, Ness Ziona, Israel.

matic pipette, and filtered through an Evans filter.[7] The filtrate was centrifuged at 3,000 r.p.m. for three minutes, the supernatant was discarded, and the sedimented cells were washed once in Hank's salt solution. The concentration of the suspension was then determined with the aid of a hemocytometer; it was diluted in growth medium to give a final concentration of 1×10^6 cells/ml. The final suspension was distributed into sterile culture tubes. For observation of the cytopathogenic effect of the virus under the microscope, 1.0-ml. amounts of cell suspension were added to each tube; the tubes were then stoppered with rubber stoppers, sloped, and incubated at 37 C. When virus activity was to be measured by color change[10] or by the appearance of hemagglutinins, the tubes were inoculated with 0.5 ml. of the cell suspension and were incubated in an upright position.

Titrations and Neutralization Tests.—Serial dilutions of NDV were prepared in maintenance mediums and 0.1-ml. aliquots of the appropriate dilutions were inoculated into the allantoic cavity of embryonating eggs or into tissue cultures. The eggs or tissue cultures were incubated for various periods, as described in the following sections, and the presence or absence of virus infection was then determined. Titers were calculated in accordance with the method of Reed and Muench[18] and were expressed as log e.l.d.$_{50}$ for eggs or log t.c.i.d.$_{50}$ for tissue culture per 0.1 ml. of virus suspension.

For neutralization tests, immune serums were mixed with equal volumes of tenfold dilutions of NDV in maintenance medium. After incubation of the serum-virus mixture in a 37 C. bath for one hour, 0.1-ml. aliquots of the mixture were inoculated into the allantoic chamber of 10-day-old embryonating eggs or into tissue cultures. At least five eggs or tissue culture tubes were used for each dilution. Virus titrations were carried out in parallel using known nonimmune serums. Results were expressed in log neutralization index, *i.e.*, log l.d.$_{50}$ (or i.d.$_{50}$) per 0.1 ml. of mixture (virus plus nonimmune serum), minus log l.d.$_{50}$ (or i.d.$_{50}$) per 0.1 ml. of mixture (virus plus immune serum).

RESULTS

Titration of Newcastle Disease Virus in Tissue Cultures and in Embryonating Eggs.—(1) Titrations Depending on the Cytopathogenic Effect.—The cytopathogenic effects of viruses on tissue cultures can be utilized for titration of virus in tube cultures in one of two ways: (1) by inoculation of the cultures with serial dilutions of the virus followed by direct microscopic observation of cell destruction or (2) by indirect evidence of such destruction indicated by the presence of, or lack of, color change of the pH indicator in the medium.[19] Changes in the pH of the medium could not be successfully used for indicating infection with NDV. The chicken embryo cells, predominantly fibroblasts, rapidly acidified the medium (pH 6.4) and degenerated due to this acidity.

Maintaining the cultures in an atmosphere of 4 per cent CO_2 or adding "tris" buffer up to inhibitory concentrations did not keep the pH above 6.4 to 6.6 in most cultures for more than 48 to 72 hours after inoculation of the virus. In cultures which were successfully maintained for longer periods, destruction of cells was evident microscopically only after three days in tubes inoculated with high concentrations of the virus and after four to five days in tubes inoculated with terminal dilutions. As a result, the medium in most cultures became acid even in the presence of virus and the color change could not be used as a criterion of the presence or absence of infection. Cultures which became acid showed degenerative changes, even in the absence of virus, and this led to confusion in reading the results of infection.

Attempts were made to reduce the rate of acid production by adjusting the concentration of cells in the suspension. This could be achieved only when the cell concentration was accurately adjusted within a narrow range, making this method impractical. Next, the amount of glucose in the medium was reduced to delay acid formation. One-half milligram per milliliter of glucose was found to be ample to initiate multiplication and to prevent acidification of the medium but the cells died by the end of 72 hours. This was apparently due to the utilization of the glucose by the cells more rapidly than necessary for their maintenance.

That such was the case was demonstrated by the following experiment: A cell suspension in M1 medium was distributed into culture tubes. After 24 hours of incubation, the medium was replaced by maintenance medium containing only 0.1 mg./ml. of glucose (M2). After 48 hours of additional incubation, 0.1 mg. of glucose was added to each of a group of tubes. On the following day, the tubes which had received additional glucose showed cells normal in appearance, whereas the cells in other tubes had degenerated completely. On the next day (*i.e.*, the 5th day from the beginning of incubation), 0.1 mg. of glucose was again added to each tube. In this manner, it was possible to maintain the cells in good condition and without acidification of the medium for five to six days,

although only 0.3 mg. of glucose had been added to each milliliter of culture. The same amount of glucose, supplied to the culture on the first day, would not have sufficed to keep the cells viable for 72 hours. The substitution of M2 medium for M1 after 24 hours of incubation and the addition of 0.1 mg./ml. of glucose to each tube after every 48 additional hours of incubation made it possible to carry out consistent titration of NDV in the culture tubes by the method of direct microscopic observation of cell destruction.

Because of the obvious drawbacks of this method, attempts were made to replace the glucose in the maintenance medium with other energy-supplying compounds. In the presence of sodium pyruvate at concentrations of 1.2 to 2.4 mg./ml., the cells multiplied but degenerated after 48 hours. Sodium lactate in concentrations of 1.2 to 2.4 mg./ml., even with the addition of small amounts of glucose to initiate growth, was also unsuitable. Sodium acetate in a concentration of 2.44 mg./ml., with the addition of 0.1 mg./ml. of glucose, gave satisfactory results and cultures could be maintained in such acetate medium (M3) for four to five days without difficulty and without change in pH.

Titrations of NDV in cultures, using M2 and M3 as maintenance mediums, were performed in the following manner: Cell suspensions were prepared as usual in medium M1. After 24 hours of incubation at 37 C., M2 or M3 were substituted for M1 and the tubes were inoculated with 0.1 ml. of virus, diluted in the corresponding maintenance medium. After four to five days of additional incubation at 37 C., the results of the virus titration were determined according to cell degeneration. At the same time, parallel titrations were carried out in embryonating eggs. Results obtained in the tissue cultures showed no significant differenc from those obtained in embryonating eggs (table 1).

TABLE 1—Time of Appearance of Cytopathogenic Action of Newcastle Disease Virus in Tissue Culture

Virus dilution	Tube No.	Days after infection			
		2	3	4	5
10^{-6}	1	N	D	D	D
	2	ND	D	D	D
	3	N	ND	D	D
	4	N	N	ND	D
	5	N	ND	ND	D
10^{-7}	6	N	N	N	D
	7	N	N	N	D
	8	N	N	N	D
	9	N	N	N	D
	10	N	N	N	D
10^{-8}	11	N	N	N	ND
	12	N	N	N	ND
	13	N	ND	ND	ND
	14	N	N	N	N
	15	N	N	D	D
10^{-9}	16	N	N	N	N
	17	N	N	N	N
	18	N	N	N	N
	19	N	N	N	N
	20	N	N	N	N
Control	21	N	N	N	N
	22	N	N	N	N
	23	N	N	N	N
	24	N	N	N	N

N = cells normal in appearance; ND = normal-appearing and degenerated cells seen in the culture; D = all cells degenerated.

Cells were grown in M1 medium for 24 hours. The M1 was then replaced with maintenance medium M3 and the cultures were inoculated with 0.1 ml. of diluted virus.

2) Titrations Based on the Appearance of Hemagglutinins.—The above methods of titration entailed both considerable work, microscopic examination of each culture, and time, four to five days, for the reading of final results.

Therefore, another method, similar in certain respects to that described by Gajdusek [10] in which the development of hemagglutinins rather than cell destruction served as a criterion of infection, was used in the following experiments:

Tissue cultures were prepared as usual, except that tubes received 0.5 ml. of cell suspension and were incubated in an up-

TABLE 2—Changes with Time in the Results of Reading Titrations of Newcastle Disease Virus in Tissue Cultures

Experiment No.	Medium	No. of tubes per dilution	Dilution factor	Log titer ($t.c.i.d._{50}$)* after days				Day of highest titer
				2	3	4	5	
275	M1	6	10.0	8.29	8.34	—	—	2 and 3
027	M1	12	5.0	8.15	8.12	7.7	—	2
057	M2	8	3.2	7.67	8.06	8.28	7.93	4
155	M3	4	10.0	7.0	7.32	7.66	—	4
215	M3	12	10.0	—	7.7	8.0	7.75	4
275	M3	15	10.0	—	—	7.83	7.5	4
276	M3	8	10.0	7.2	7.8	7.8	7.5	3 and 4
275	M4	5	10.0	6.0	7.0	7.7	7.6	4

— = not tested.

* $t.c.i.d._{50}$ = 50 per cent tissue culture infective dose.

right position. When M1 or M4 mediums were to be used throughout the experiment, the tubes were infected with serial dilutions of NDV at the time of preparation of the cultures. When mediums M2 or M3 were to be used, the tubes were infected 24 hours later, *i.e.*, at the time of replacement of M1 by M2 or M3. After two to five days of incubation, 0.2 ml. of a 1 per cent suspension of washed chicken erythrocytes in saline were added to each culture tube and, following one hour of incubation in the cold (4 to 8 C.), the results of tests for hemagglutination were read by the pattern method.[2] The presence of cells and cell debris on the bottom of the tubes did not interfere with the formation of the button of erythrocytes when no agglutination took place, or with the formation of a typical diffuse hemagglutination pattern in the case that sufficient virus propagation had occurred.

The results of experiments designed to find the appropriate time for reading the test (*i.e.*, for determining the presence of hemagglutinins) are shown (table 2) and the results of titrations in cultures maintained in various mediums as compared to those obtained by titration in embryonating eggs are summarized (table 3). Using M2, M3, or M4 mediums, the highest titers were obtained when the cultures were tested on the fourth day. Comparable titers were obtained in M1 medium when the cultures were tested after 48 to 72 hours. In all experiments, the titers in tissue cultures were consistently lower than in embryonating eggs by a factor of two to eight.

Neutralization Tests.—Neutralization tests with serums of chickens immune to NDV were performed in embryonating eggs and in tissue cultures using M1, M2, M3, and M4 mediums. Hemagglutinin formation was the criterion of infection. When M1 or M4 mediums were to be used, the serum-virus mixtures were added at the time of preparation of the cultures and the results were read after 48 to 72 hours of incubation. When M2 or M3 were to be used, the serum-virus mixtures were added 24 hours later, at the time of replacement of M1 by M2 or M3, and the results were read after four days of additional incubation. In the case of M2, 0.05 mg. of glucose was added to each tube (1 drop of a 1.0 mg./ml. of solution of glucose in Hank's balanced salt solution) after every 48 hours of incubation. Immune and control chicken serums were used in dilutions 1:10, 1:100, and 1:1,000. The results of these experiments are summarized (table 4). In serum diluted 1:10, M1, M2, and M4 mediums gave results almost identical with those

TABLE 3—Comparative Titrations of Newcastle Disease Virus in Eggs and Tissue Cultures as Determined by the Appearance of Hemagglutinins

Experiment No.	Day of reading of test	No. of eggs or tubes per dilution	Dilution factor	Log titer per 0.1 ml. of virus suspension in					Ratio: $\frac{\text{e.l.d.}_{50}}{\text{t.c.i.d.}_{50}}$
				Eggs (e.l.d.$_{50}$)	Tissue culture (t.c.i.d.$_{50}$) in				
					M1	M2	M3	M4	
113	5	5	5.0	8.5	.....	.....	8.32	.....	1.5
094	5	5	2.0	4.55	.....	.....	4.17	.....	2.4
094	5	5	2.0	4.8	.....	.....	4.36	.....	2.8
134	6	6	5.0	8.45	.....	.....	7.82	.....	4.2
174	5	5	10.0	8.0	.....	.....	7.6	.....	2.5
075	4	4	10.0	8.08	.....	.....	8.68	.....	0.4
215	4	12	10.0	8.5	.....	.....	8.0	.....	3.16
276	4	8	10.0	8.24	.....	.....	7.8	.....	2.7
147	4	5	10.0	8.37	.....	.....	8.0	.....	2.3
Average ratio									2.44
254	5	5	10.0	8.33	.....	7.4	.....	.....	8.4
155	4	5	10.0	8.8	.....	7.66	.....	.....	13.0
057	4	8	3.2	8.62	.....	8.28	.....	.....	2.2
Average ratio									7.8
215	3	12	10.0	8.5	7.6	.....	.....	.....	8.0
275	3	12	10.0	8.95	8.24	.....	.....	.....	4.0
027	2	12	5.0	8.62	8.15	.....	.....	.....	3.0
147	2	5	10.0	8.37	7.37	.....	.....	.....	10.0
Average ratio									6.2
147	3	5	10.0	8.37	.....	.....	.....	7.85	3.3
257	4	5	10.0	8.5	.....	.....	.....	7.7	5.6
Average ratio									4.4

TABLE 4—Serum-Neutralization Tests of Newcastle Disease Virus in Tissue Culture and in Embryonating Eggs

Experiment No.	Serum No.	Reciprocal of serum dilution	Log neutralization index as determined in				
			Embryonating eggs	Tissue culture in medium of			
				M1	M2	M3	M4
155	3	10	5.0	....	5.0	5.0	....
174	3	10	5.0	....	....	7.5	....
154	3	10	5.0	....	5.0	....	....
		100	4.0	....	3.0	....	....
		1,000	1.0	....	0	....	....
075	3	10	4.6	....	....	7.5	....
146	4	10	5.8	5.3	....	....	....
		100	4.1	3.3	....	....	....
147	4	10	5.2	5.8	....	7.4	5.5
		100	3.5	2.0	....	3.4	2.4
		1,000	0.6	0.1	....	0	0.7

obtained in embryonating eggs. On the other hand, the neutralization index, measured in M3 medium, was at least two logs higher and an end point could not be reached in some cases. In 1:100 dilution of the serum, M3 gave results equal to those obtained in eggs, whereas in M1, M2, and M4 neutralization indexes were 1.0 to 1.5 logs lower.

DISCUSSION

The estimation of virus titer based on the statistical outcome of infection of tissue cultures or embryonating eggs is obviously less accurate than a method in which individual virus particles show up as plaques, as in the method of Dulbecco.[5] However, the first method is technically simpler for use in the ordinary bacteriological laboratory and is sufficiently accurate for most practical purposes. Furthermore, when used for neutralization tests, the former method allows the results to be expressed in the form of the neutralization index in a manner comparable to the results of tests performed in embryonating eggs or animals. Since the development of neutralizing antibodies in vaccinated birds serves as a reliable index of the development of immunity to NDV, a relatively simple and inexpensive method for measuring the effectiveness of NDV vaccines by neutralization tests is of obvious importance. The use of tissue cultures in test tubes instead of embryonating eggs makes the titration much more economical, since 40 to 60 cultures—the equivalent of a like number of eggs for these purposes—can be prepared from a single embryo, and 500 to 600 tubes can be easily made up by one technician during a working day. In order to determine infection by cell degeneration, it was found necessary to maintain the chicken fibroblasts for at least five days in viable condition, since the HP strain of NDV required four to five days to exert its full cytopathogenic effect. In the usual tissue culture medium containing 1.0 mg./ml. of glucose, these cells died because of rapid production of acid. Only when the glucose concentration was reduced to 0.1 mg./ml. and an additional 0.1 mg. of glucose was added to each milliliter of culture every 48 hours could the cultures be maintained for six days in viable condition, their pH remaining above 6.8. The same amount of glucose, if added in bulk at the beginning of incubation, would not have sufficed to keep the cells alive for 72 hours. Glucose could be replaced by sodium acetate but not by sodium pyruvate or sodium lactate. Titrations carried out in glucose medium (M2) or in the acetate medium (M3), using the method of microscopic observation of cell degeneration, gave results identical with those carried out in the allantoic cavity of embryonating eggs.

In a second method of virus titration in tissue culture, the formation of hemagglutinins in infected tubes was utilized. These titrations, carried out in embryonating eggs, gave titers slightly but consistently higher than those obtained in tissue cultures with any of the four mediums used. The sensitivity of tissue culture to infection thus seemed to be lower than that of intact chorioallantoic membrane.

Neutralization tests with immune chicken serums, performed in tissue cultures in four different mediums, gave results identical with those obtained in embryonating eggs. Mediums M1 and M4 showed an advantage over M2 and M3 in that the total time required for the test in

the former two was 48 to 72 hours and no change of medium was necessary, whereas M2 and M3 required a change of medium and incubation of five days until results could be read.

Several investigators[6,21] have pointed out that the neutralization index can be expected to vary inversely with the sensitivity of the host to infection, *i.e.*, the more sensitive the host, the lower the neutralization index. The results described above, using mediums M1, M2, and M4 failed to show this relationship and the converse was found when highly diluted serum was used. Using M3 medium and immune serum (diluted 1:10), a neutralization index was obtained at least two logs higher in the tissue cultures than in the embryonating eggs. On the other hand, no significant difference was found between the sensitivity to infection—as reflected in results of titrations—between cultures maintained in M3 and cultures maintained in M1, M2, and M4.

The influence on the neutralization index of the tissue culture medium and the sensitivity to infection of the test system is now under investigation.

Summary

Methods utilizing monolayer tissue cultures of chicken embryo tissues for the performance of titrations and neutralization tests of Newcastle disease virus (NDV) are described. Virus titers were determined either by the method of direct microscopical observation of cell destruction or by the appearance of hemagglutinins. The former required changes of medium and four to five days of incubation, whereas the latter required, in one of the methods used, two to three days of incubation and no change of medium.

In the case of neutralization tests, hemagglutinin appearance only was used as the criterion of the presence of unneutralized virus.

Results of titrations and neutralization tests in tissue culture were found to be comparable though not identical with those found in embryonating eggs.

Chicken embryo fibroblasts were found to metabolize glucose more rapidly than necessary for cell maintenance, leading under certain conditions to cell degeneration due to excessive acidification of medium.

Sodium acetate, but not sodium pyruvate or lactate, could replace glucose in the tissue culture medium.

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The Normal Hematological Picture of Turkey Poults and Blood Alterations Caused by Enterohepatitis

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THERE have been few observations reported in the literature on the normal hematology of the turkey. Scott *et al.*[1] and Rhian *et al.*[2] reported the hemoglobin content and blood chemistry determinations of turkeys. Tables presented by Wirth[3] and Wintrobe[4] show cell numbers and hemoglobin content for normal adult turkeys. Descriptions of the circulating and bone marrow blood cells have recently been published by Lucas and Jamroz.[5]

Alterations in the differential white blood cell (w.b.c.) count of turkeys exposed to *Histomonas meleagridis* were reported by Johnson and Lang.[6] McGuire and Cavett[7] observed changes in the total red (r.b.c.) and white blood cell counts along with alterations in the blood chemistry of turkeys suffering from enterohepatitis.

MATERIALS AND METHODS

The 20 apparently healthy Beltsville White turkeys used in this study were obtained from a commercial hatchery as 1-day-old poults.

The turkeys were placed in separate units in a chicken battery. Turkeys designated as controls were placed above those which were to be inoculated, thereby lessening the chance of fecal contamination.

Heterakis gallinae ova may become infected with the etiological agent which produces blackhead. Therefore, embryonated ova of *H. gallinae* (from chicken cecums) were employed in this experiment to produce enterohepatitis in the turkeys. Twelve turkey poults, 45 days old, were inoculated orally with embryonated *H. gallinae* ova (1 ml. containing 300 to 600 ova) by means of a glass pipette. Seven of the inoculated turkey poults expired from histomoniasis. One poult recovered from the infection, and 4 poults showed no signs of infection. The diagnosis of enterohepatitis was based on characteristic symptoms, gross and microscopic lesions, and the presence of *H. meleagridis* in the tissue sections. Eight birds were used as controls. The control turkeys and those which were inoculated but survived were killed by exsanguination.

Blood samples were taken from all turkeys on the first day after inoculation and every third day thereafter until the fourteenth day; from then until death, blood was taken every second day. The blood was obtained alternately from the two wing veins in order to minimize trauma. The blood smears were stained with Wright's stain. Blood from each oxalated sample was drawn into a r.b.c.-diluting pipette, diluted 1:200 with a phloxine dye (Wiseman[8]), and allowed to stand for one hour. Erythrocytes were counted in the hemocytometer in the usual manner. Leukocytes were counted by the indirect method of Olson[9] and the total leukocyte count calculated by the following formula:

$$\text{Total leukocyte count} = \frac{10}{9} \left[\begin{array}{c} \text{No. of acidophilic cells counted in hemocytometer} \end{array} \right] \times 200 \times \frac{100}{\text{Percentage of acidophilic cells counted in blood smear}}$$

A Haden-Hauser hemoglobinometer was used to measure hemoglobin content.

RESULTS AND DISCUSSION

Normal Hematology of Turkeys.—The normal blood picture of turkeys in this experiment was based on a study of 8 poults, 6 weeks old.

Determination of the total leukocyte count presents certain difficulties due to the fact that the r.b.c. are nucleated. Three methods for determining the total w.b.c. count of fowls are the indirect (Schmeisser[10]), semi-indirect (Wiseman[8]), and the direct method of Rees-Ecker as modified by Wintrobe.[4] In the indirect method, all w.b.c. in the hemocytometer are counted and, from the stained slide, the proportion of erythrocytes and leukocytes determined. The semi-indirect method developed by Wiseman employs a phloxine stain to differentiate the w.b.c. from the r.b.c. The ratio of stained w.b.c. counted in the hemocytometer to those counted on the slide is determined. In the direct method, the number of w.b.c. in five of the 1-sq. mm.

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Taken from a thesis, "The Normal Histology and Hematology of the Turkey and the Pathology Due to *Histomonas Meleagridis*," submitted (by Malewitz) to the School for Advanced Graduate Studies of Michigan State University, in partial fulfillment of the requirements for the Ph.D. degree, June, 1956.

TABLE 1—Compilation of the Normal Blood Picture in Turkeys

Investigator	Age of poults (wk.)	r.b.c. x 1,000	w.b.c.	Hemoglobin (Gm./100 cc.)	Av. percentage of w.b.c.				
					Heterophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Johnson & Lang [6]	9	.....	.....	.....	43.4	0.9	3.2	50.6	1.9
Wirth [3]	....	2,700	34,100	.....	34.5	2.5	2.5	63.6	1.0
McGuire & Cavett [7]	8 to 18	2,476	38,700	9.3	43.2	0.1	4.6	43.6	4.9
Wintrobe [4]	....	1,930	.....	11.2	.....	.....	.....	.....	.....
Present investigation	5 to 8	2,270	30,685	10.2	29.9	1.5	6.1	61.4	1.3

squares of the counting chamber are counted, and the number of w.b.c. per cubic millimeter of blood is then calculated. Hirschfeld and Hoitink [11] reported a method similar to the Rees-Ecker method. Natt and Herrick [12] used a new diluent for the semi-indirect method for counting leukocytes. Denington and Lucas [13] compared the various methods of determining the total w.b.c. count in fowl and concluded that only slight differences occur between the three methods.

Since red granules are present in both eosinophils and heterophils, these cells may be easily confused. The granules are rod-shaped in the heterophil and spherical in the eosinophil. Natt and Herrick [14] observed an inner spherical granule within the rodlike granules of the heterophil. The addition of buffer, as used in the Wright's staining procedure, may cause a partial or complete dissolution of the rodlike granules leaving only the inner spherical granules. Denington and Lucas [13] reported variability in samples of Wright's stain which affected the staining of the blood cells. In this investigation, w.b.c. containing round, large, red-refractive granules were counted as eosinophils; those with rodlike or small red granules were designated heterophils.

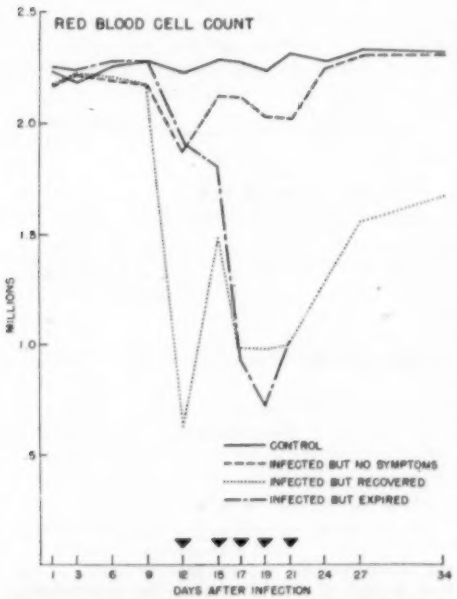

Fig. 1—Red blood cell (r.b.c.) counts of the four groups of turkeys during the experimental period. ▼ indicates r.b.c. counts which were statistically significant. The r.b.c. count of the turkeys which showed no signs of an infection was statistically significant only on the twelfth day.

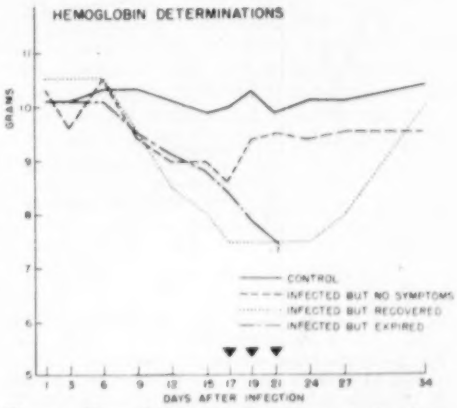

Fig. 2—Hemoglobin determinations of the four groups of turkeys during the experimental period. ▼ indicates hemoglobin determinations which were statistically significant. The hemoglobin of the turkeys which showed no signs of an infection was statistically significant only on the seventeenth day.

The average normal r.b.c. and w.b.c. counts, hemoglobin, and differential counts obtained in this study are compared with the results reported by other investigators (table 1).

Hematology of Infected Turkeys.—The 7 infected turkeys which died of histomoniasis exhibited the following average blood picture: r.b.c., 1,670,000; hemoglobin (Gm./100 cc. of blood), 9.2; w.b.c.,

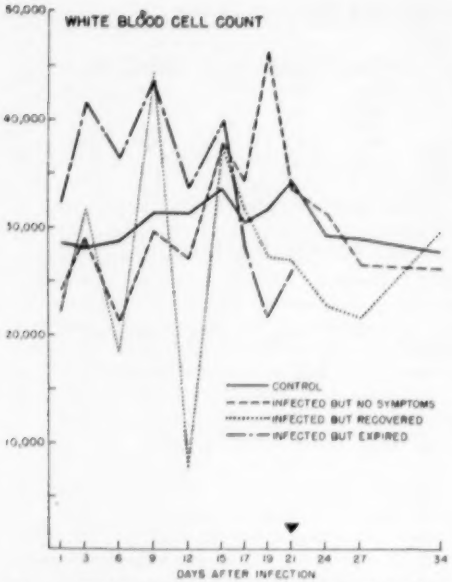

Fig. 3—White blood cell (w.b.c.) counts of the four groups of turkeys during the experimental period. ▼ indicates w.b.c. counts which were statistically significant (only for turkeys which died from histomoniasis).

35,890; heterophil, 45.4 per cent; cosinophil, 1.3 per cent; basophil, 3.7 per cent; lymphocyte, 48.5; monocyte, 1.1 per cent.

The total r.b.c. count began to decrease on postinfection day 9 and continued to drop until the death of the turkey indicating a normocytic anemia. The blood showed signs of sludging from approximately postinoculation day 17 until death. An accurate trend of the r.b.c. count during this period could not be made because of the clumped cells. The hemoglobin content decreased from postinoculation day 6 until death. The total w.b.c. count of infected turkeys increased between postinoculation days 3 and 15 and then decreased until the time of death. McGuire and Cavett [7] observed that the leukocyte count rose at the time of death to three times that of the controls. A decrease (from 32 to 22 %) of heterophils was observed between postinoculation days 3 and 6. From postinfection days 6 to 9, the heterophil count appeared slightly above the average of that of the control birds. The heterophil count steadily increased after day 12 and, at death, was four times greater than that of the control turkeys.

Previous investigators (Johnson and Lang,[6] McGuire and Cavett [7]) reported a similar increase in heterophils. A lymphocytosis occurred between postinoculation days 3 and 9. Lymphopenia persisted from postinfection day 12 until the time of death. Johnson and Lang [6] and McGuire and Cavett [7] observed this same trend.

The blood picture of the turkey which recovered from the disease displayed a trend similar to that of the birds which died of histomoniasis. The total r.b.c. count, after an initial decrease, began to increase on postinfection day 21; the hemoglobin decreased between postinoculation days 6 to 24, after which it increased, and when this bird was killed the hemoglobin had returned to normal; the total w.b.c. count decreased between postinoculation days 3 to 6, increased between days 6 and 9, decreased from days 9 to 12, rose on day 15, then decreased until day 27,

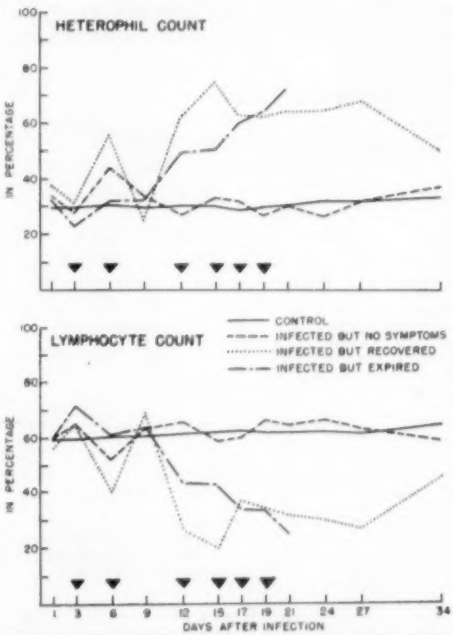

Fig. 4—Heterophil and lymphocyte counts of the four groups of turkeys during the experimental period. ▼ indicates white blood cell counts which were statistically significant. The heterophil and lymphocyte counts of turkeys which died from the disease were statistically significant on days 3, 12, 15, 17, and 19. The heterophil and lymphocyte counts on infected turkeys showing no signs of an infection were statistically significant only on day 6.

and approached the count of the control birds at the time of exsanguination. The heterophils decreased on postinoculation day 3; increased to approximately two and a half times that of the controls from postinoculation days 12 to 27, and approached that of normal birds at the time of exsanguination. A lymphopenia was exhibited from postinoculation days 9 to 34, at which time the bird was killed.

The total r.b.c. count and hemoglobin of the 4 birds which were inoculated but showed no signs of disease were slightly lower than that of the controls. The total w.b.c. count varied slightly more than in the normal birds (20,000 to 45,000/cmm. of blood). No change was observed in the heterophil or lymphocyte count.

A comparison of the blood pictures observed in the four groups of turkeys can be made from the data compiled (fig. 1 to 4). The averages are based on 7 birds from postinfection days 1 to 15, 6 from days 15 to 19, 5 on day 21, and 1 on day 24. No apparent changes between inoculated and control birds were observed in the eosinophil, basophil, and monocyte counts.

The blood counts of infected birds which died and infected birds showing no signs of infection were compared with the control birds. Analysis of variance was employed to test the statistical significance of the blood counts in these groups (fig. 1 to 4).

Summary

The hematology of 9 uninfected turkeys, 5 to 8 weeks old, was studied and compared with the results of other investigators.

Of 12 turkeys inoculated with embryonated *Heterakis gallinae* ova to produce a Histomonas infection, 7 died from the disease, 1 recovered, and 4 showed no signs of infection.

Blood studies of the turkeys which later died from histomoniasis revealed a decrease in the total red blood cell (r.b.c.) count, hemoglobin, and white blood cell (w.b.c.) count. The heterophils increased while the lymphocytes decreased. Monocytes, eosinophils, and basophils remained within a normal range.

The turkey which recovered from the infection showed a decrease in the total r.b.c. count, hemoglobin, and total w.b.c. count and an increase in heterophils. All counts approached those of the control turkeys at the time of exsanguination except for the lymphocytes, where a lymphopenia persisted.

Four exposed turkeys showed no signs of infection but exhibited a slightly lower total r.b.c. count and hemoglobin content, and a more variable total w.b.c. count than the control birds. No apparent change in the differential counts of this group was observed.

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Immunity Study of Three Types of Newcastle Disease Vaccine for Broilers and Caponettes

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BROILER PRODUCERS in Rhode Island need a method for the early protection of their flocks against Newcastle disease (ND). It is generally believed that vaccination in early life produces a short period of immunity which usually necessitates revaccination. This experiment was planned to compare the degree and length of immunity produced by employing the three types of vaccines most commonly used.

MATERIAL AND METHODS

Vaccines.—The three types of commercial ND vaccines used were: water (Lasota strain), dust (B_1 strain), and killed* (California strain 11914). They were purchased from a local feed company.

Chicks.—The chicks used were obtained from the poultry department of the University of Rhode Island. The parent flock had been vaccinated with ND wing-web vaccine seven months before the eggs were incubated. Six lots of chicks were used (table 1). Lots 5 and 6, of 70 chickens each, were given the killed vaccine and used as controls, respectively. They were housed in separate pens on the university farm. The four lots of 50 chickens each, which were used for water and dust vaccine trials, were housed in separate pens, about 200 feet apart, on an isolation farm 2 miles from the University Poultry Farm.

Vaccination.—All vaccines were prepared and administered as recommended by the manufacturer. Water and dust vaccines were first administered when the birds were 5 days old. The killed vaccine was given intramuscularly (0.5 ml.) at 10 days of age.

At 4 weeks of age, 50 chickens were taken from both the water- and dust-vaccinated lots and each of these groups were then housed separately in pens 50 feet apart. They were revaccinated with water or dust vaccines, respectively.

Vaccinated chickens were kept under satisfactory brooding and feeding conditions during the experiment.

At 3 weeks of age and each week thereafter, 5 chickens were taken from each of the vaccinated lots, as well as from the control lot. These sample birds were identified, bled for hemagglutination-inhibition testing (HI), and challenged. The lots that were revaccinated at 4 weeks of age were treated similarly when they were 6 weeks old.

Antigenic response to vaccination was determined by the *beta* HI test. Lederle's diagnostic antigen and method were used. Eight units of antigen were used and the serum dilutions were 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128.

The challenge strain of ND virus used was the G.B. Texas 1948.[2] This strain is highly virulent and the symptoms produced are mainly paralysis, or death, or both. The inoculum for challenge consisted of 0.25 ml. of pooled infected allantoic fluid dropped into the trachea of each bird. The titer of the allantoic fluid was 10^7 e.l.d.$_{50}$. Birds were closely observed for two weeks and were held for eight weeks following the challenge. The interpretation of immunity was based on the presence of paralysis, or death, or both.

The challenged birds were kept in an isolated pen and were cared for by a special attendant who had no contact with the rest of the experimental chickens.

Laboratory Procedures.—These trials were carried out to determine the longevity of the dust and water vaccine strains of ND virus in the environment after vaccination.

Trial 1, Water Vaccine.—Immediately after dis-

Contribution 894 of the Rhode Island Agricultural Experiment Station, Kingston.

The authors express their appreciation to Dr. Vance J. Yates, head of the Animal Pathology Department, University of Rhode Island, for his valuable help in editing this paper.

* Formalin-inactivated, aluminum hydroxide-adsorbed vaccine.

TABLE 1—Plan of Experiment

Group	No. of chickens	Type of vaccine	Age at 1st dose	Age at 2nd dose	No. of birds challenged/ week/group	Age at* challenge (wk.)
1	50	Water	5 days	.....	5	3 to 12
2	50	Dust	5 days	.....	5	3 to 12
3	50	Water	5 days	4 weeks	5	6 to 15
4	50	Dust	5 days	4 weeks	5	6 to 15
5	70	Killed	10 days	.....	5	3 to 16
6	70	None	.....	.....	5	3 to 16

* The 5 birds challenged each week in the six groups were the same age and were challenged at the same time.

TABLE 2—Hemagglutination-Inhibition Titers of Chicks After Vaccination for Newcastle Disease with Water Vaccine when 5 Days Old

Age in weeks									
3	4	5	6	7	8	9	10	11	12
....	....	....	....	....	....	....	....	....	....
4	....	....	....	....	....	....	....	....	....
16	....	....	....	....	....	....	....	....	....
16	....	....	....	4	....	....	4	....	....
16	....	....	....	4	....	....	8	....	4

solving the vaccine in water and providing the chickens with it, a small amount of water was transferred into four sterile rubber-capped tubes which were placed in test tube racks in the chicken house. Samples were taken from these tubes 24, 48, 72, and 96 hours from the time the vaccine was administered. An inoculum of 0.15 ml. containing 5.0 mg. of streptomycin was inoculated into a set of 6, 10-day-old chicken embryos.

Trial 2, Dust Vaccine.—Sixteen Petri dishes in sets of four were used in this trial. Respective plates in each set, containing 1.0 Gm. of dried droppings, shavings, feathers, or feeding mash, were sterilized with their contents and placed, uncovered, under the hover while the chickens were dusted. The Petri dishes were covered after dusting. At 24 hours and each day for four days, one of each kind was removed and its contents were tested for the presence of virus. The contents of the Petri dishes were dissolved in 5.0 ml. of sterile physiological saline solution. This suspension was cleared by centrifugation and an inoculum of 0.15 ml. was injected into a set of six eggs. The inoculum contained 5.0 mg. of streptomycin. Two serial passages were then made in 10-day-old chicken embryos.

Parental Immunity.—In order to determine the parental immunity status of the 5-day-old chicks which were used in the experiment, serum was secured from the control chicks at five days and tested using the serum-neutralization method of Cunningham.[4]

RESULTS

Vaccination with Water Vaccine Strain (Lasota Strain) at Five Days.—In this trial, the chicks showed a "take" the day after the vaccine was administered. This take was in the form of sneezing and sniffing. The reaction did not last more than four days. The HI titer at three weeks was noticeable and 4 birds had titers of 4, 16, 16, and 16, respectively (table 2). The HI titers dropped sharply between the fourth and twelfth weeks. During this period, only 5 birds had titers of 8, 4, 4, 4, and 4; the rest of the birds were negative. Challenge indicated that at the end of 12 weeks 28 per cent of the birds were resistant (table 8).

TABLE 3—Hemagglutination-Inhibition Titers of Chicks After Vaccination for Newcastle Disease with Water Vaccine when 5 Days and 4 Weeks Old

Age in weeks									
6	7	8	9	10	11	12	13	14	15
....	....	8	4	8	8	16	4	....	....
....	....	8	16	8	16	16	4	4	4
....	....	16	16	16	32	32	8	64	8
....	....	32	16	32	32	64	16	64	32
....	....	32	16	64	64	64	32	64	32

Vaccination with Water Vaccine (Lasota Strain) at Five Days and Four Weeks.—In this group, the HI antibody titers were negative at six and seven weeks (table 3).

TABLE 4—Hemagglutination-Inhibition Titers of Chicks Vaccinated for Newcastle Disease with Dust Vaccine when 5 Days Old

Age in weeks									
3	4	5	6	7	8	9	10	11	12
....	....	....	....	....	4	4	....	....	....
....	....	....	....	....	8	8	4	4	....
4	8	4	32	8	16	16	8	4	....
4	8	8	32	16	64	16	8	8	....
8	16	16	64	64	64	32	8	8	4

At eight weeks, the titers were between 8 and 32. They rose steadily through the twelfth week but were on the decline by the fifteenth week. The highest HI titer reached during this period was 64. Of the birds challenged, 94 per cent were immune (table 8).

Vaccination with Dust Vaccine (B_1 Strain) at Five Days.—The chicks showed a respiratory reaction in the form of a mild rasping sound after they were dusted with the B_1 vaccine. It appeared that only a few chickens had a take starting four days after vaccination and lasting about a week. Of the 50 chicks vaccinated, 34 (68 %) had HI antibody titers which varied between 4 to 64 (table 4). Starting the sixth and through the ninth week, the titers were relatively high and then they started to drop. Four birds had titers of less than 4, at 12 weeks. Of the birds challenged, 70 per cent were resistant (table 8).

Vaccination with Dust Vaccine (B_1 Strain) at Five Days and Four Weeks.—The revaccination at 4 weeks of age with the dust vaccine caused a noticeable HI titer response after the sixth week. Two birds at 6 weeks of age were negative and the other 3 had titers of 16, 16, and 32, respectively (table 5). The birds that were bled the ninth and through the eleventh week had an HI titer of 128 (the highest

TABLE 5—Hemagglutination-Inhibition Titers of Chicks Vaccinated for Newcastle Disease with Dust Vaccine when 5 Days and 4 Weeks Old

Age in weeks									
6	7	8	9	10	11	12	13	14	15
....	32	32	128	128	128	8	16	4	4
....	32	32	128	128	128	8	16	32	8
16	64	64	128	128	128	16	32	32	8
16	64	128	128	128	128	32	128	64	64
32	128	128	128	128	128	128	128	128	64

serum dilution tested). Although the antibody titer decreased gradually after the eleventh week, at the end of 14 weeks 1 bird still had a titer of 128. All 50 birds involved in this trial resisted the challenge virus (table 8).

Vaccination with One Dose of Killed Vaccine at Ten Days of Age.—The range of the HI titers was 128 to 64 from the third through the eleventh week, following which it continued to decrease gradually (table 6). The HI titer could still be detected in all the birds through the sixteenth week. The percentages of immune chickens at the end of 15 and 16 weeks were 100 and 98.6, respectively.

Control Group.—None of the control birds showed a positive HI titer or proved to be immune to challenge.

Results of Laboratory Procedures.—The results of the trials 1 and 2 indicated that it was possible to recover the virus in the samples of the water vaccine after 24 and 36 hours, but not after 72 hours.

With the dust vaccine, it was not possible to recover the virus after 24 hours in any of the dried droppings, shavings, feathers, and feeding samples.

Parental Immunity.—The results of the serum-neutralization test showed that at five days serum from the control chicks neutralized 100 units of G.B. Newcastle disease virus.

DISCUSSION

The life span of broilers and caponettes is relatively short. Producers usually market these chickens when they are between 9 and 16 weeks old. During the growing period, broilers and caponettes are debeaked, given medication, one or two vaccines, and hormonized. Occasionally, if vaccination is not handled carefully, the birds may continue to show respiratory symptoms and may come down with signs of chronic respiratory disease or other air sac infections. A condition described sometimes as "overvaccination" is also blamed for bringing about such conditions. The results of this experiment may indicate how vaccines can be used in the field most effectively.

TABLE 6—Hemagglutination-Inhibition Titers of Chicks Vaccinated for Newcastle Disease with Killed Vaccine when 10 Days Old

Age in weeks														
3	4	5	6	7	8	9	10	11	12	13	14	15	16
128	64	64	64	64	64	64	32	64	32	8	8	4	4
128	64	64	64	64	64	64	32	16	8	4	4	4	4
128	64	64	64	64	64	64	32	32	8	8	4	4	4
128	64	64	64	64	64	64	32	64	32	8	8	4	4
64	64	64	64	64	64	64	64	64	32	8	8	4	4

As the result of one dose of water vaccine, at 5 days of age, the chickens showed a reaction two days after vaccination, and HI antibody titers at three weeks. However, this response was relatively poor when measured by HI and challenge tests. At three weeks, 4 of 5 birds had HI antibodies. Only 18 per cent of the birds in the group were HI positive.

Four of 5 birds (80 %) challenged at three weeks were immune. However, the immunity decreased sharply starting the fourth week and, at the end of 12 weeks,

TABLE 7—Age when Tested and Number and Percentage of Birds Positive to the Hemagglutination-Inhibition Test

Kind of vaccine	Age in weeks														No. of birds	Percentage
	Age given															
	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
Water at 5 days	4	0	0	0	2	0	0	2	0	1	....	....	....	....	9	18
Water at 5 days and 4 weeks	....	....	....	....	....	5	5	5	5	5	5	4	4	....	38	76
Dust at 5 days	3	3	3	3	3	5	5	4	4	1	....	....	....	....	34	68
Dust at 5 days and 4 weeks	....	....	....	3	5	5	5	5	5	5	5	5	5	....	48	96
Killed vaccine at 10 days	5	5	5	5	5	5	5	5	5	5	5	5	5	5	70	100
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TABLE 8—Number and Percentage of Birds Resistant when Challenged

Kind of vaccine	Age in weeks														No. of birds	Percentage
	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
	Age given															
Water at 5 days	4	1	0	1	3	1	1	2	1	0	....	....	....	....	14	28.0
Water at 5 days and 4 weeks	....	....	....	2	5	5	5	5	5	5	5	5	5	....	47	94.0
Dust at 5 days	3	4	3	3	3	5	5	4	4	1	....	....	....	....	35	70.0
Dust at 5 days and 4 weeks	....	....	....	5	5	5	5	5	5	5	5	5	5	....	50	100.0
Killed vaccine at 10 days	5	5	5	5	5	5	5	5	5	5	5	4	5	5	69	98.6
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

only 28 per cent of the group withstood challenge.

Administration of a second dose of water vaccine at four weeks brought a noticeable increase in the HI titers as well as in the immunity of the chickens. During the sixth and seventh weeks, HI antibodies were not detected. The HI antibody titers did not exceed a dilution of 64. A relatively large number of birds (76%) demonstrated HI antibodies. In this group, 94 per cent of the chickens were resistant to challenge.

The group of chickens given one dose of dust vaccine responded with HI antibody titers which prevailed for a longer period than in the group given one dose of water vaccine (tables 5 and 7). In this group, 68 per cent of the birds were HI positive. At the end of 12 weeks, 70 per cent were immune.

Of the chickens given two doses of dust vaccine, 2 birds (40%) were HI negative during the sixth week. In this group, 44 per cent of the birds reached HI antibody titers of 128. This finding agrees with Beninato *et al.*[1] who demonstrated a favorable response to initial vaccination for ND in 13 of 17 flocks using mixed ND and infectious bronchitis dust vaccines. They also showed an increase in HI antibodies following treatment.

In the group that was vaccinated with aluminum hydroxide-adsorbed, killed ND virus, the birds showed high HI antibody titers until 12 weeks of age. These titers reached their highest level of 128 dilution when the chicks were 3 weeks of age. At the end of the tenth week, 57 per cent had 128 to 64 HI titers. Later in the experiment, the titers declined gradually. At the eleventh and twelfth weeks, 14 per cent had titers of 64 to 8 and 28 per cent had titers of 8 to 4 at 13 and 16 weeks of age. In this group, all the chickens resisted challenge until 14 weeks of age. At the end of 15 weeks, only 1 bird succumbed to challenge. Similar results of the partial protection value of killed ND vaccine were demonstrated by Brandly *et al.*,[2] Hanson *et al.*,[5] Waller and Gardener,[9] and Hofstad.[6–8]

Under the conditions of this experiment, the use of one dose of water or dust vaccine did not produce sufficient protection for broilers. This was especially true where the water vaccine was used. The administration of two doses of either water or dust vaccines induced satisfactory immunity. Such results were as good as those found in birds vaccinated with one dose of killed vaccine at 10 days of age.

SUMMARY

Six groups of chickens, totaling 340, were used to determine the immunity response to three types of commercial Newcastle disease (ND) vaccine.

Of the group (70 birds) vaccinated with aluminum hydroxide-adsorbed, killed ND vaccine at 10 days of age, 98.6 per cent were immune to challenge at 16 weeks of age.

Of the birds given one dose of water vaccine when 5 days old, 28 per cent resisted challenge at the end of 12 weeks, but their immunity was poor at 4 weeks of age. Revaccination at four weeks acted as a "booster" and 94 per cent of the 50 birds in this group were immune when 15 weeks old.

Of the 50 birds given one dose of dust vaccine when 5 days old, 70 per cent were immune at the end of 12 weeks. A group of 50 birds was given the dust vaccine

when 5 days and again when 4 weeks old. All of these showed immunity at 15 weeks. They possessed a greater immunity and higher hemagglutination-inhibition (HI) antibody titers at 15 weeks of age than the group treated once.

The water and dust vaccine viruses survived in the chicken environment for 36 and 24 hours, respectively.

The serum from 5-day-old control chicks neutralized 100 units of G.B. ND virus.

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The Bacterial Flora of the Respiratory Tract of Chickens Affected with Chronic Respiratory Disease

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IT IS generally recognized that chronic respiratory disease (CRD) is a definite poultry disease entity and that pleuropneumonia-like organisms (PPLO) are the specific causative agent. However, under field conditions, it is not always possible to isolate PPLO from birds with CRD. Bacteriological cultures from the exudates from the sinus, trachea, and air sacs, as well as from the liver and pericardium of these birds, frequently result in the isolation of a variety of bacteria other than PPLO.

Osteen's [1] report, in 1954, indicated that in many typical air sac respiratory infections pure cultures of *Escherichia coli* were obtained and were considered to be the sole infective agent. Cover and Waller,[2] while studying CRD in pipped eggs, made close observations of a severely infected parent flock. Cultures of the lungs and air sacs of the parent birds yielded staphylococcic, streptococcic, and *coli-aerogenes* organisms. However, PPLO were observed in material taken from these birds for embryo inoculation.

A detailed laboratory analysis of one month's respiratory consignments by Luginbuhl *et al.*[3] revealed a high number of isolations of bacteria and fungi. Cultures on standard agar slants of cardiac blood, liver, spleen, lungs, and exudates from 75 birds resulted in the isolation of coliform organisms 48 times and of fungi from lungs and exudates, 16 times.

Bacteriological examinations by Fahey [4] of exudates from air sacs, liver, and pericardium of 187 chickens with CRD resulted in the isolation of 85 cultures of Proteus, 131 cultures of *Esch. coli*, 50 cultures of Pseudomonas, 44 cultures of Aerobacter, 73 cultures of Micrococcus, 43 cultures of Streptococcus, 20 cultures of Salmonella, and 21 cultures of other bacteria. Most of the specimens examined were in the terminal stages of infection, which may account for the high number of Escherichia, Proteus, and Pseudomonas isolations.

The objective of this investigation is to determine the bacterial flora of chickens with CRD raised under field conditions.

MATERIALS AND METHODS

A total of 51 chickens with CRD from eight flocks was submitted for bacteriological culturing. Twelve chickens from two flocks, free of CRD, were selected as controls. When the birds were killed, the cultures were made immediately if possible, if not, they were kept at ordinary refrigerator temperature. These birds were discarded if the cultures were not made within 12 hours.

Feathers were moistened with a 1:5,000 roccal® solution prior to the opening of the body cavity which was accomplished by cutting through the ribs on each side and on through the coracoid and clavicle bones. Using sterile swabs or a bacteriological inoculating loop, exudates from the sinus, trachea, air sacs, as well as swabs of the liver and pericardium, were cultured on the following mediums: *(a)* bacto-PPLO agar (1:2,000 concentration of thallium acetate) enriched with approximately 1 per cent bacto-PPLO serum; *(b)* tryptose blood agar plates; *(c)* bacto-Levine eosin methylene blue agar; *(d)* bacto-SS agar; and *(e)* B-B-L thioglycollate broth.

The inoculated PPLO agar plates were incubated at 37 C. for 120 hours; all the other culture mediums were incubated at 37 C. for 48 hours. During the incubation period, bacto-PPLO agar plates were examined microscopically (x 100) for minute, round colonies with dense centers and a less dense periphery as described by Lecce and Sperling.[5]

Whenever colonies fitting the above description were found on PPLO agar plates, they were removed by cutting the agar around them into blocks with a sterile scalpel. Two to four such agar blocks, approximately 2 to 3 mm. square, were removed from each plate. One block of agar was dropped into a tube of bacto-PPLO broth for cultural and biochemical studies of PPLO. This broth, enriched with 1 per cent bacto-PPLO serum fraction, was made from the same basic ingredients as bacto-PPLO agar except that the additional agar and thallium acetate were omitted. The second block of agar was subjected to the Diene's [6] staining technique.

Two additional blocks of agar were removed when

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excessive contamination of the original bacto-PPLO agar was noted. One block of agar was dropped into a tube of bacto-PPLO enriched broth. This medium was designed to suppress the growth of both gram-positive and gram-negative contaminants by the addition of crystal-violet (0.1 Gm./liter). The second additional agar block was inverted upon a bacto-PPLO agar plate on which 0.05 ml. of a solution containing 50,000 units of penicillin per milliliter had been dropped. All of the inoculated mediums were incubated at 37 C. for 120 hours or until colonies resembling PPLO were observed.

Smears made from suspected colonies of PPLO were stained with concentrated Giemsa solution for 30 to 40 minutes. These smears were examined microscopically for the characteristic small pleomorphic bacilli or coccoid bodies.

Eosin methylene blue agar plates were examined for the presence of Escherichia or Aerobacter colonies. Colonies considered to be typical of Escherichia and Aerobacter were transferred to nutrient or tryptose broth for further studies consisting of the following differential tests: *(a)* methyl red; *(b)* Voges-Proskauer; *(c)* Kovac's test for the production of indole; *(d)* Koser citrate medium; and *(e)* carbohydrate fermentations (the production of acid and gas by the coliform-*aerogenes* group was determined by inoculating the following phenol red carbohydrate broths: saccharose, sorbitol, inulin, lactose, dextrose, maltose, esculin, galactose, xylose, raffinose, arabinose, dulcitol, salicin, trehalose, inositol, and levulose).

Bacto-SS agar plates were examined for transparent or colorless smooth colonies that are characteristic of the Shigella and Salmonella groups.

Tryptose blood agar plates were used because of their suitability for the cultivation and isolation of many fastidious pathogenic bacteria. Hemolytic and nonhemolytic staphylococcic organisms were isolated and pathogenicity determined by their ability to produce coagulase (coagulase test), an enzyme capable of clotting plasma. Other organisms growing on tryptose blood agar plates were identified by routine morphological and biochemical studies.

Gram stains were made on the bacterial growth appearing in thioglycollate broth. Whenever necessary for identification purposes, subcultures were made to tryptose blood agar plates, bacto-Levine eosin methylene blue agar, and bacto-SS agar.

RESULTS

Twenty-nine (57%) of the 51 chickens with CRD were found to be harboring Escherichia sp. in one or more tissues. These organisms were found in tissue and tissue exudates (table 1) in the following frequency: trachea, 56.8 per cent; air sac, 41.1 per cent; sinus, 11.7 per cent; pericardium, 11.7 per cent; and liver, 1.9 per cent. Escherichia sp. were found in the trachea of all 29 birds and 21 (72.0%) were shedding these bacteria in both the trachea and air sac. Tracheal exudates (table 1) from 2 chickens yielded *Aerobacter aerogenes* organisms.

Because of the large number of Escherichia sp. organisms isolated from trachea and air sacs of chickens with CRD, physiological studies were conducted on 21 selected strains. Fifteen strains were classified as *Esch. coli*, three strains as *Escherichia freundii*, and two strains as *Escherichia intermedium*; one strain could not be classified. Profuse growth was obtained when coliform organisms were subcultured on bacto-PPLO agar containing serum fraction and thallium acetate. These organisms appeared as short rods (resembling cocci) when stained for 30 minutes with Giemsa's solution. However, their morphological characteristics were similar, except for the larger size, to the coccoid forms of PPLO.

Nonhemolytic staphylococci that failed to produce coagulase (negative coagulase test) were from tissue and tissue exudates

TABLE 1—Bacterial Isolations from Exudates of 51 Chickens Infected with Chronic Respiratory Disease (CRD)

Organism	Sinus		Trachea		Air sac		Pericardium		Liver		Total No. of isolations
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	
PPLO *	0	0	7	13.7	0	0	0	0	0	0	7
Escherichia sp.	6	11.7	29	56.8	21	41.1	6	11.7	1	1.9	63
Nonhemolytic staphylococci	5	9.8	12	23.5	13	25.5	0	0	0	0	30
Hemolytic staphylococci	0	0	2	3.9	2	3.9	0	0	0	0	4
Pseudomonas sp.†	1	1.9	6	11.7	3	5.8	0	0	0	0	10
Unclassified streptococci	1	1.9	2	3.9	1	1.9	0	0	0	0	4
Proteus sp.	1	1.9	3	5.8	1	1.9	0	0	0	0	5
Yeastlike	2	3.9	1	1.9	0	0	0	0	0	0	3
Gram-positive bacilli	0	0	3	5.8	0	0	0	0	0	0	3
Aerobacter aerogenes	0	0	2	3.9	0	0	0	0	0	0	2
Salmonella sp.	0	0	0	0	1	1.9	0	0	0	0	1

* Morphologically similar to pleuropneumonia-like organisms. † Six strains of *Pseudomonas aeruginosa*, four strains of Pseudomonas sp.

of 14 birds. A total of 30 isolations (table 1) were made from the sinus, trachea, and air sac exudates; the largest number of isolations (a total of 13) was made from the air sacs. Hemolytic staphylococci that produced coagulase (positive coagulase test) were found in the trachea and air sacs of 2 chickens with CRD. These coagulase-positive strains of staphylococci were considered to be pathogenic.

Pseudomonas sp. organisms were isolated from 11.7 per cent (table 1) of the tracheal exudates and 5.8 per cent of the air sac exudates. Six of the ten isolated strains were considered to be *Pseudomonas aeruginosa*.

An organism resembling a species of the genus Proteus was isolated from tracheal exudates (table 1) of 3 chickens. Additional isolations were also made from the sinus and air sac of 1 chicken.

A gram-negative bacillus considered to be Salmonella sp. was isolated from the air sac exudate (table 1) of 1 chicken. This organism produced smooth colorless colonies on bacto-ss agar and failed to ferment bacto-phenol red lactose broth.

Yeastlike organisms (table 1) of uncertain classification were isolated from sinus and tracheal exudates of 3 infected birds.

Tracheal exudates from 3 chickens were found to be harboring large gram-positive bacilli (table 1) of no apparent significance.

Colonies of PPLO were observed on bacto-PPLO agar plates (table 1) swabbed with tracheal exudates from 7 (13.7%) chickens. These colonies, when observed microscopically (x 100), appeared as small target-like structures, similar to those previously described by Lecce and Sperling.[5] When stained with concentrated Giemsa's solution, the organisms were small pleomorphic rods and cocci. However, when blocks of agar containing these colonies were dropped into bacto-PPLO broth in a manner previously described, profuse growth of coliform organisms was observed in five of the seven inoculated bacto-PPLO broth tubes. Smears made from the remaining two broth cultures failed to reveal PPLO after an incubation period of seven days.

Highly contaminated bacto-PPLO agar blocks, when dropped into tubes containing bacto-PPLO broth with added crystal-violet, failed to produce detectable bacterial growth. Pleuropneumonia-like organisms were not observed in smears made from the inoculated broth.

On two occasions, contaminated bacto-PPLO agar blocks, inverted on bacto-PPLO agar plates, on which 0.05 ml. of a solution containing 50,000 units of penicillin per milliliter had been dropped did not completely inhibit the growth of contaminants.

Unsuccessful attempts were made to decolorize suspected colonies of PPLO stained in a manner originally described by Diene and recommended by Lecce and Sperling.[5] Control colonies of staphylococci and *Esch. coli* organisms, grown on bacto-PPLO agar without inhibitors, were also stained by Diene's technique. The staphylococci were decolorized within 30 minutes, while colonies of *Esch. coli* were only partially decolorized.

Escherichia sp. were isolated from the trachea of 2 (16.6%) of the 12 chickens selected as CRD-free control birds. A yeastlike organism was observed in cultures made from the trachea and air sacs of 1 bird. A nonhemolytic, coagulase-negative Staphylococcus was found on tryptose blood agar plates swabbed with exudates from the trachea and air sacs of 1 control chicken.

SUMMARY

The bacterial flora of the respiratory tract of 51 chickens affected with chronic respiratory disease (CRD) was studied in detail. Escherichia sp. organisms were isolated from the exudates of 29 (57.0%). Of the 21 strains of Escherichia sp. originally isolated from the diseased birds, 15 were classified as *Escherichia coli*, 3 as *Escherichia freundii*, and 2 as *Escherichia intermedium;* one strain of Escherichia sp. could not be classified.

Nonhemolytic and hemolytic Staphylococcus, *Pseudomonas aeruginosa*, Pseudomonas sp., Proteus, Aerobacter, Salmonella, Streptococcus, gram-positive bacilli, and yeastlike organisms were also isolated from the exudates.

Tracheal exudates from 7 chickens were harboring bacteria morphologically and culturally similar to pleuropneumonia-like organisms (PPLO); considerably fewer than that normally expected, due to profuse coliform contamination.

Thallium acetate, when added to PPLO agar in a concentration of 1:2,000, failed

to inhibit the growth of coliform organisms isolated from exudates of diseased chickens.

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The Role of Turkey Eggs in the Transmission of Ornithosis

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SINCE the initial recognition of ornithosis in turkeys in 1948 by Irons *et al.*,[1] there have been several other known outbreaks of this disease in Texas, New Jersey, and Oregon turkeys from which virulent ornithosis virus was isolated. This disease has been recognized in other areas by serological studies and isolation of strains of low pathogenicity. There has been much speculation as to the natural spread of ornithosis to susceptible turkeys. Aside from the theories that various carriers, vectors, and fomites spread the disease, transmission of turkey ornithosis via hatching eggs has been strongly suspected by many workers.

There are no records of experiments which show that ornithosis or psittacosis is transmitted from the hen via the egg to the offspring. Meyer [2] suggested egg transmission or nestling transmission in psittacine birds. He was able to recover the virus from ovaries and from egg yolk in the oviduct of parakeets but actual egg transmission was not demonstrated.

Davis and Vogel [3] found that their strain of psittacosis virus when inoculated into chicken embryos of various ages could be isolated from some of the hatched chicks. When embryos less than 8 days of age were inoculated, isolation of virus from hatched chicks was unsuccessful.

Davis [4] examined 70 pigeon eggs for psittacosis virus without recovering the agent. Fritzsche *et al.*[5] stated that ornithosis virus could not be isolated from eggs of infected pigeons. Davis and Delaplane [6] found no evidence of egg transmission of ornithosis during a natural outbreak or in a limited experimental study in turkeys.

The purpose of this paper is to report the results of more extensive studies on egg transmission of ornithosis in turkeys.

MATERIALS AND METHODS

Experiment 1.—On Nov. 21, 1955, 150 Beltsville Small White turkey hens, 9 months old, and 15 toms were obtained from the Texas A. & M. Poultry Farm. The turkeys were placed in a fenced lot on the Veterinary Research Farm. They were fed a commercial, breeding mash without medication. Upon arrival, a blood sample was drawn from each turkey for complement fixation inhibition (CFI) tests. Four months later (March 8, 1956), all the turkeys were bled again for CFI studies.

On March 19, 1956, when the egg production rate reached 30 per cent, each hen was inoculated intratracheally with virulent ornithosis virus (Jo strain). The inoculum consisted of 0.25 ml. of pooled fifth passage amnioallantoic fluid from 30 chicken embryos inoculated on the seventh day and dead on the eleventh day. The toms were exposed to ornithosis by contact with the hens.

On postinoculation days 3, 5, 7, 9, 12, and 15, blood samples were taken from 5 hens to determine the possibility of viremia. On May 15, 1956, and during September, all survivors were bled for postinoculation CFI tests.

Necropsies were performed on the birds which died and on all the others when they were killed, six months after inoculation. The lesions were recorded and spleens collected individually for virus-isolation attempts in chicken embryos.

Because 10 toms died from the infection, 4 toms which had had experimental ornithosis the preceding year were placed in the pen with the hens four weeks following inoculation in an attempt to maintain a satisfactory fertility rate in the eggs.

Eggs laid after the hens were inoculated were collected daily, marked, and set in the incubator. On the seventh day of incubation, the eggs were candled and the infertile ones were discarded. Those containing live embryos were candled daily thereafter until the embryos either died or hatched. On the day an embryo died, it was assayed for the presence of virus. Watery yolk and the whole embryo were collected from eggs containing dead embryos during the second week of incubation. Yolk, amnioallantoic fluid, and embryo viscera were collected from those that died during the third and fourth week of incubation. Streptomycin was used at the rate of 25.0 mg. per milliliter of original inoculum to control bacterial contamination when necessary. Two-tenths milliliter of the

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This work was conducted in cooperation with the Agricultural Research Service, U. S. Department of Agriculture.

The authors extend their appreciation to J. E. Neal, D.V.M., and A. L. Burroughs, Ph.D., serologists, for conducting the complement fixation inhibition tests.

Presented before the Combined Sections on Poultry and Research, Ninety-Third Annual Meeting, American Veterinary Medical Association, San Antonio, Texas, Oct. 15–18, 1956.

preparation was inoculated via the amnioallantoic route into each of 5, 7- or 8-day-old chicken embryos, which were observed for nine days. Amnioallantoic fluid from all chicken embryos which died was checked for the presence of bacteria; when negative, a blind passage was made into another series of embryos.

On the twenty-fifth day of incubation, eggs containing live embryos were placed in a hatching incubator in a separate room. The poults which hatched were wing-banded and brooded in electric battery brooders. They were placed on the floor at 5 to 6 weeks of age. Later, they were moved into a newly constructed, isolated range pen where poultry had not been raised. This procedure created a multiple-aged brooding and growing program. The spleen of each first-generation turkey that was killed or died was suspended in nutrient broth and assayed for virus in chicken embryos. On Oct. 25, 359 turkeys were in the flock and a blood sample was drawn from each poult for CFI tests.

Experiment 2.—Of 200 turkey eggs obtained locally, 100 were inoculated with ornithosis virus and set in the incubator. The inoculum was freshly prepared, egg-propagated, amnioallantoic fluid from a sixth passage of Jo strain ornithosis virus which had an e.i.d.$_{50}$ at 10^{-6} for 7-day-old chicken embryos. The amnioallantoic fluid was diluted with nutrient broth and 0.1 ml. was placed into the yolk of each egg. Fifty eggs received a 10^{-6} dilution and 50 received a 10^{-7} dilution. After seven days of incubation, the infertile eggs and dead embryos were individually assayed for virus, using chicken embryos. The eggs containing live embryos were candled daily thereafter until the embryos died or hatched.

The 100 uninoculated eggs served as controls for the first seven days of incubation. The eggs containing live embryos on the seventh day were divided in two groups. Thirty-three embryos received a 10^{-5} dilution and 32 received a 10^{-6} dilution of ornithosis virus which titered at an e.i.d.$_{50}$ at 10^{-6} for chicken embryos. Each embryo was inoculated via the amnioallantoic route with 0.1 ml. of diluted virus. The eggs were candled daily thereafter until the embryos died or hatched. All embryos which died were individually assayed for virus. Poults which hatched were killed at 2 weeks of age and virus-isolation attempts were conducted on the spleens.

Experiment 3.—Five turkey hens in production were inoculated intratracheally with egg-propagated Jo strain ornithosis virus. All eggs were collected daily thereafter until production ceased. Each day, approximately one half of the eggs laid were assayed individually for virus. Yolk and albumen were collected from each egg. Approximately 0.5 ml. of the mixture was inoculated into the yolk sac of each of 5, 7-day-old chicken embryos. The other eggs were incubated for 24 hours before they were assayed in a similar manner.

Blood was collected on postinoculation days 3, 4, 5, 6, and 15 for the purpose of determining viremia. Necropsies were conducted on the hens that died and on those killed on the fifteenth day. Ovaries were assayed in chicken embryos for virus.

Experiment 4.—This experiment dealt with the possibility of transmission of ornithosis virus on the egg shell. Turkey egg shells were broken in small pieces and sterilized in an autoclave. These shells were immersed for five minutes in amnioallantoic fluid and egg yolk containing ornithosis virus. Approximately 1.0 Gm. of impregnated shells was placed in each of six sterile cotton-plugged jars. The jars were placed in an egg incubator operating at a dry bulb reading of 99 F. and a wet bulb reading of 87 F. Daily thereafter, a jar was removed and the egg shells were washed in 5.0 ml. of nutrient broth. Two-tenths milliliter of this wash was inoculated into each of 5, 7-day-old chicken embryos via the amnioallantoic route. As a control for this experiment, cotton swabs were substituted for egg shells and treated in a similar manner.

RESULTS

Experiment 1.—Signs of disease first observed on the fifth postinoculation day persisted in the hens for approximately two weeks. They included depression, yellowish to green diarrhea causing stained vent feathers, pyrexia, respiratory distress with rattling, anorexia, and cessation of egg production. The first sick tom was observed 14 days after the hens were inoculated. The 4 toms which were added to the infected flock did not develop clinical evidence of ornithosis.

Virus was recovered from the blood of turkeys in the flock from postinoculation days 7 to 15.

Thirty-five (23.3%) of the hens died from postinoculation days 7 to 18. Ten (66.7%) of the toms died from days 17 to 21 after the hens were inoculated. The lesions varied in severity and were similar to those previously reported in turkeys infected with Jo strain ornithosis virus.[7]

Egg production continued normally for six days after inoculation; it dropped sharply on the seventh day and ceased after the ninth. A total of 312 eggs was collected before production ceased; of which total, 15 eggs were laid during the known viremic stage of the disease. Of the 312 eggs, 115 were infertile, 17 contained dead embryos, and 180 contained live embryos on the seventh day of incu-

TABLE 1—The Results of Incubating and Assaying Eggs Laid by Turkeys Experimentally Infected with Ornithosis

No. set	7th day observation			Embryo mortality—Days of incubation																				Poults hatched	Virus isolation attempts
	Inf.	Dead	Fer.	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
312*	115	17	180	20	11	6	7	4	5	1	3	1	1	3	1	2	1	1	1	0	0	0	0	68	44	59
2,642†	677	148	1,817	36	32	29	36	38	31	16	35	22	16	12	6	11	12	3	14	15	43	47	1	776	586	577
Totals 2,954	792	165	1,997	56	43	35	43	42	36	17	38	23	17	15	7	13	13	4	15	15	43	47	1	844	630	636

Inf. = infertile; Fer. = fertile.
* Eggs collected before hens went out of production. † Eggs collected after hens came back in production.

bation. During the remainder of the incubation period, 136 embryos died and 44 poults hatched. Eight of these poults died and the survivors were killed at 2 weeks of age. Ornithosis virus was not recovered in 59 attempts from either turkey embryos or poults.

The hens remained out of production for 19 days. One egg, laid 29 days after inoculation, was fertile and the embryo died on the twenty-seventh day of incubation. From postinoculation days 29 to 42, the egg production rate increased to about 45 per cent, remained constant for about six weeks, then gradually declined to zero by day 178. After the hens returned to production, 2,642 eggs were collected of which 677 were infertile. Of the others, the embryos in 148 were dead by the seventh day of incubation, 1,231 embryos died during further incubation, and 586 poults hatched. Ornithosis virus was not recovered from these dead embryos or from 198 poults which died or were killed. Occasionally, tissues of several embryos were pooled; however, 577 isolations were attempted of which 73 were blind passed. The number of turkey eggs collected, the results of incubation, and the number of virus isolation attempts conducted are are shown (table 1).

The serums of 359 poults, taken on Oct. 25, were negative to the CFI tests. The results of CFI tests on the serums of the turkeys from the parent flock are shown (table 2).

Necropsy of the parent hens which were killed revealed that most of them had fibrous adhesions within the pericardial sac, in the mesentery, and on the surface of the liver—usually on the left latero-ventral side. Apparently, these were residual lesions of ornithosis. Peritonitis was observed in a few hens but, in each instance, there was evidence of reproductive tract disorder. Lesions observed in the toms were fibrous adhesions within the pericardial sac and localized fibrosis of the liver capsule. Two of the 4 toms added to the flock showed no gross lesions. All spleens appeared normal in size and texture. An attempt to isolate virus from each spleen was unsuccessful.

Experiment 2.—The results of inoculating turkey eggs are shown (table 3). Ornithosis virus was not recovered from infertile eggs. Of 67 fertile eggs, virus was recovered from 3 dead embryos on the seventh day, from 1 on the eighth day, and 1 on the ninth day but no virus was recovered after that.

The mortality increased when 7-day-old turkey embryos were inoculated. Virus was recovered from 43 of 61 dead embryos; however, no recoveries were made past the sixteenth day of incubation.

Experiment 3.—The 5 inoculated hens laid 23 eggs before production ceased; four were laid on the first day, five on the second, three on the third, four on the fourth, three on the fifth, one on the sixth, and three on the seventh. Virus was not recovered from any of these eggs of which 15

TABLE 2—The Results of the Complement Fixation Inhibition Test of Serums from Turkeys Experimentally Infected with Jo Strain Ornithosis Virus

Date	No. tested	No. of complete positive reactions at indicated serum dilution								Positive (total)	Positive (%)
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256		
Nov. 21, 1955	165	3	0	1	0	0	1	0	0	5	3
March 8, 1956	165	0	0	0	0	0	0	0	0	0	0
March 19, 1956—INOCULATED											
May 15, 1956	120	3	5	13	10	12	7	5	10	65	54
September, 1956	103	6	11	15	11	15	14	4	7	83	81

were not incubated and 8 were incubated.

A viremia was detected in all 5 hens on the third to sixth postinoculation days and in 1 hen on the fifteenth day.

Signs of ornithosis were first noticed on the fourth day and persisted until the survivors were killed on the fifteenth day. Two hens died on the ninth day. Lesions observed were similar to those of an acute ornithosis infection, as previously reported.[7] The ovaries were degenerated. Ornithosis virus was recovered from the ovaries of the 2 hens that did not have a viremia when killed.

Experiment 4.—The Jo strain ornithosis virus failed to survive more than three days on egg shells or four days on cotton swabs in the incubator.

DISCUSSION

From these studies, it appeared that Jo strain ornithosis virus was not transmitted to poults hatched from eggs laid by turkey hens in the viremic or postrecovery stages of ornithosis. The daily collection and incubation of turkey eggs should have favored the possibility of recovering virus since loss of the agent due to storage was minimal.

In the first experiment, all infertile eggs on the seventh day of incubation were discarded. The advisability of this was questioned; therefore, in the second experiment, all infertile eggs were assayed but no virus was recovered. In the first experiment, negative results were obtained when all embryos dead by the seventh day were assayed. The same procedure was followed in the second experiment but, this time, virus was recovered from some of the dead embryos.

Successful recovery of virus seemed to be influenced by the duration of time that the dead embryo remained in the incubator before being assayed. Ornithosis virus did not survive in infertile eggs at 99 F. for seven days, which may be explained, in part, by the fact that the virus did not survive for more than four days on cotton swabs in the egg incubator. Page[8] stated that the New Jersey strain of ornithosis virus would not survive for more than 48 hours at 37 C.

Since the agent appeared to be labile under egg-incubator conditions for seven days, it was decided to assay turkey eggs sooner. Fresh eggs were assayed in the third experiment but virus could not be recovered. It seemed reasonable to expect that virus should have been present in the eggs because virus was recovered from blood and ovaries of the infected hens. Dilution of virus in the egg contents beyond the sensitivity of the assay method may have been the reason for failure to recover the agent.

Some workers contend that latency of ornithosis virus accounts for the clinical disease when stress is applied. Stress on the embryos caused by malposition due to nonrotation of the eggs was evidenced by high embryo mortality on the twenty-eighth day. The hatching procedures used in this experiment should be considered as stress because some poults were kept which otherwise would have been killed. The poults were subjected to stress by

TABLE 3—Embryo Mortality and Virus Recovery by Days of Incubation Following Inoculation of Turkey Eggs with Ornithosis Virus

	No. eggs	Inoculum dilution	7th day observation			Embryo mortality—Days of incubation																					Poults hatched
			Inf.	Dead	Fer.	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
	50*	10^{-6}	18	10	22	2	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2	15
Virus recovery			0	3	..	1	0	..	..	..	..	..	..	..	..	..	0	..	..	..	..	..	..	..	..	0	0
	50*	10^{-7}	15	7	28	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	19
Virus recovery			0	0	..	0	1	0	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0	0
	50†	10^{-5}	12	5	33	0	1	1	2	2	10	11	3	2	0	0	0	0	0	0	0	0	0	0	1	0
Virus recovery			..	..	..	..	0	0	2	2	10	11	3	1	..	..	..	..	..	..	..	..	..	..	..	0	..
	50†	10^{-6}	13	5	32	5	4	3	4	4	3	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	4
Virus recovery			..	..	..	1	0	0	2	4	3	3	0	1	..	..	..	..	..	..	..	..	..	..	..	..	0

Inf. = infertile; Fer. = Fertile.

* Inoculated prior to incubation. † Inoculated 7-day-old embryos which had served as controls for the first seven days of incubation.

using multiple-age brooding and growing procedures.

It has been shown that poults are highly susceptible to ornithosis virus; mortality is usually above 80 per cent in 3- to 4-week-old poults and the infection will spread among turkeys in contact with each other.[6, 7, 9] Thus, if ornithosis virus were egg transmitted, clinical disease would be manifested in young poults hatched from eggs produced by infected hens.

SUMMARY

Virus was not recovered from dead embryos or poults obtained from eggs laid by breeder turkeys experimentally infected with the Jo strain of ornithosis.

When fresh turkey eggs were inoculated with ornithosis virus, the agent was not recovered beyond the ninth day of incubation. When 7-day-old turkey embryos were inoculated, the mortality was high and ornithosis virus was not recovered beyond the sixteenth day.

Ornithosis virus was not recovered from eggs laid by hens in the viremic stage of ornithosis when isolation attempts were made on freshly laid and 24-hour incubated eggs.

Ornithosis virus lived no longer than three days on turkey egg shells or four days on cotton swabs under egg-incubator conditions.

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"Atomized" Distemper Vaccine of Avian Origin
I. Experimental Immunization of Ferrets and Mink

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THE PRACTICAL APPLICATION of wet spray or dust vaccines has been unequivocally demonstrated by their successful use in the mass vaccination of chickens against Newcastle disease and infectious bronchitis.[1,2] Similar methods of vaccination would be of real value, both from the economic and labor standpoints, were they available for the immunization of other species of animals bred in large numbers on confined premises. Morris *et al.*[3] applied a wet spray of chicken embryo-adapted distemper virus to susceptible ferrets and reported rapid protection against the disease under specified conditions. Similarly, Gorham and associates[4-6] immunized ferrets with a nebulized distemper vaccine and demonstrated the applicability of the method to the experimental vaccination of young and adult mink. In addition, Gorham *et al.*[7] obtained successful preliminary results with a dust distemper vaccine in ferrets and mink under strict experimental conditions.

The present communication reports the results of experimental vaccination of ferrets and mink with either dust or wet "atomized" distemper vaccines, as measured by direct challenge or by the complement-fixation (CF) method.

MATERIALS AND METHODS

Virus Strains.—The American Cyanamid (Lederle) strain of chicken embryo-adapted distemper virus[8] was used for the preparation of vaccines as described below. Challenge of vaccinated and control ferrets was carried out with the American Cyanamid (Lederle) stock strain of virulent distemper virus[8] by the intraperitoneal route, each animal receiving between 100 and 1,000 minimum lethal dose (m.l.d.).

Preparation of Vaccines.—The atomized dust vaccine was prepared by the method of Markham *et al.*[1] for Newcastle disease and infectious bronchitis viruses, except that infected embryonic tissue rather than allantoic fluid was used. The dried tissue suspensions were blended in a proportion of 1:5 in aluminum silicate. The atomized aqueous vaccines were made by one of two methods: (1) dust vaccine was resuspended in sterile water, centrifuged to pack the aluminum silicate, and the supernatant used for the vaccination of animals; (2) infected embryonic tissues were treated by the method described in the accompanying paper.[10]

Susceptible Ferrets.—These were procured as previously described[11] and maintained under the same conditions of strict isolation.

Mink.—The animals used in this study were adult sapphire mink at least 1 year of age at the time of vaccination.

Vaccination Procedures.—These are described individually for each experiment recorded below.

Complement-Fixation Method.—The CF technique employed is essentially the Kolmer-Boerner test,[12] which utilizes overnight icebox incubation. All serums are inactivated at 56 C. for 30 minutes, and serial twofold dilutions are made in normal saline from 1:4 to 1:256. Two additional sets of 1:4 and 1:8 dilutions are also prepared. Addition of 2 units of canine distemper antigen to the tubes of the first set, an equivalent dilution of normal tissue antigen to the second set, and normal saline to the third set follows. After distribution of 1.5 full units of complement, incubation is carried out at 4 C. for about 18 hours. Two per cent sheep blood cells and two units of hemolysin are then added, and a reading is made after further incubation for 15 minutes at 37 C.

Since the preparation of antigens is essential for the success of the test and the reliability of results, it is being described here in detail. Twenty per cent suspensions of distemper-infected and normal ferret spleens are made in sterile distilled water. The preparations are distributed in convenient amounts into 100-ml. centrifuge tubes containing some sterile glass beads. They are shell-frozen and vacuum dried. The dried material, in tightly rubber-stoppered tubes, can be stored for several months at 4 C. without loss of antigenicity.

For working antigens, a certain number of tubes are extracted by pouring benzene directly on the dried tissue: two and one-half to three times the original volume. The tissues are thoroughly broken up and suspended in the benzene by shaking. The stoppered tubes are left at room temperature for one hour, with occasional mixing, and then centri-

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The authors express their appreciation to Mrs. M. Murrin and Mr. H. Klaver for their able technical assistance and to Mrs. E. Osborn for her help in the preparation of the manuscript.

fuged at 2,000 r.p.m. for ten minutes. The benzene is aspirated and discarded, replaced with fresh solvent, and the operation is repeated for a total of three or four times.

After the last centrifugation, the precipitate is frozen in the same tubes and dried overnight under vacuum. On the following day, the extracted powder is resuspended to original volume with a mixture of equal amounts of neutral buffered glycerine and 0.1 M phosphate buffer at pH 7.0, containing formalin (0.1%).

After an overnight rehydration period at 4 C., tubes of the same suspension are pooled by means of a pipette, leaving the beads behind, and the pools are submitted to a final centrifugation at 3,000 r.p.m. for 30 minutes, preferably in a refrigerated centrifuge. The clear supernatants are drawn off, and the antigenic titer of the distemper preparation is determined by the checkerboard method, in the presence of a known canine distemper-immune serum. As reported previously,[13] the presence of glycerine greatly enhances the stability of the antigen at 4 C.

Experiments in Ferrets

Atomized Dust Vaccine.—Twelve ferrets were placed in a 10-gal. galvanized iron pail, the cover of which was tightly sealed with tape except for a "dusting" hole. They were sprayed with 12 doses of dust vaccine lot 2, equivalent to 12 doses of injectable mink vaccine. Dusting was carried out by means of a Holmes spray apparatus, and the animals were kept in contact with the spray cloud for 30 minutes. When the animals were returned to their respective cages, heavy white rings of aluminum silicate were seen around the nostrils of each of them.

The following controls were also included in this test: 2 ferrets were injected intraperitoneally with 1 ml. of the original fresh suspension used for the preparation of dust vaccine, which had been kept frozen until then, and 2 more animals received, by the same route, 1 ml. of the supernatant of rehydrated dust vaccine. In addition, 2 normal ferrets were placed as contact controls with the dusted animals, and 2 others with those which were injected with the fresh-frozen tissue suspension.

Beginning on the first day and up to 11 days following exposure, pairs of dusted ferrets were challenged every other day along with pairs of normal animals as controls. The rest of the animals were similarly challenged on the eleventh day. The results (table 1) were disappointing in that all dusted ferrets developed the disease and died, as did the contact and challenge control animals. In contrast, both groups of injected animals were found to be immune, indicating that the dust vaccine contained viable virus at all stages of preparation.

Dust Versus Aqueous Atomized Vaccines.—Failure to protect ferrets by dusting led to the application of the same atomized vaccine as both a dust and an aqueous spray as follows. Using the same vaccine lot as above, 6 ferrets were dusted by the method described, except that dusting was carried out in a hermetically taped egg "pullman" box. On the same day, 6 additional ferrets were sprayed with the aqueous supernatant of the dust vaccine obtained by wetting with sterile water a quantity of dust equivalent to that used above and by centrifuging at 2,000 r.p.m. for ten minutes to pack the aluminum silicate. Spraying was also applied in a taped egg pullman box, at the rate of 1 ml. per animal, by means of a DeVilbiss 40 nebu-

TABLE 1—Results of Distemper Immunization of Ferrets with "Atomized" Dust Vaccine

Group No.	No. of ferrets	Method of vaccination	Interval between exposure and challenge (days)	Results of challenge
1	2	Dust	1	Died 15 and 16 days
1a	2	None	Controls for group 1	Died 13 and 14 days
2	2	Dust	3	Died 14 and 16 days
2a	2	None	Controls for group 2	Died 13 and 16 days
3	2	Dust	5	Died 14 and 15 days
3a	1	None	Controls for group 3	Died 14 days
4	2	Dust	7	Died 15 and 15 days
4a	2	None	Controls for group 4	Died 15 and 19 days
5	2	Dust	9	Died 17 and 17 days
5a	2	None	Controls for group 5	Died 18 and 18 days
6	2	Dust	11	Died 15 and 18 days
7	2	None	Contacts with group 6	Died 14 and 15 days
8	2	Fresh suspension, intraperitoneally	11	Survived 21 days
9	2	None	Contacts with group 8	Died 15 and 18 days
10	2	Rehydrated dust, intraperitoneally	11	Survived 21 days
10a	2	None	Controls for groups 6 to 10	Died 15 and 18 days

TABLE 2—Results of Distemper Immunization of Ferrets with Dust and Aqueous "Atomized" Vaccines

Group No.	No. of ferrets	Method of vaccination	Interval between exposure and challenge (days)	Results of challenge
1	6	Dust	23	Died between 12 and 13 days
2	6	Rehydrated dust, spray	23	Survived 21 days
3	2	Rehydrated dust, intraperitoneally	23	Survived 21 days
4	2	None	Contacts with group 3	Died 12 and 12 days
4a	2	None	Controls for groups 1 to 4	Died 13 and 14 days

lizer * under 12 lb. of laboratory live-air pressure. The animals were left in the box for an additional 20 minutes before they were removed to their cages. Two additional ferrets were injected intraperitoneally with 1 ml. of the same suspension and placed with 2 normal contact control animals.

Results of challenge of the 16 ferrets, which was done 23 days later after the inclusion of 2 challenge control animals, are given (table 2). Again, dusted animals died of the infection as did the contact and challenge controls, whereas sprayed and injected ferrets were found to be immune.

Confirmative Evidence for Atomized Aqueous Vaccine.—Vaccine lot 6368–30A was prepared by the method described in the accompanying paper.[10] It was intended for the aqueous spray vaccination of mink but, before its use in this latter species, it was tested for potency in ferrets as follows: following reconstitution of the freeze-dried vaccine with sterile water, 6 ferrets were sprayed under the same conditions as in the previous experiment, and 2 additional animals received the same preparation intraperitoneally in 1-ml. amounts. Thirteen days later, the 8 vaccinated and 2 normal control ferrets were challenged. The former remained normal, whereas the latter contracted the disease and died (table 3).

Application of Atomized Aqueous Vaccine by Hand Sprayer: The successful experiments with atomized aqueous vaccine reported above were all conducted with the DeVilbiss 40 nebulizer, which creates a mist of an average particle size of about 0.5 to 1.0 μ. However, the application of vaccine by that method is somewhat elaborate, requiring constant and relatively high pressure to expel the desired volume within a practical length of time, and rather cumbersome equipment.

In the present experiment, an attempt was made to determine whether a simple, mechanical hand sprayer could be equally successful. The Z and W sprayer † which was selected for this purpose produces particles of 75.0 to 100.0 μ and larger. A preliminary calibration having revealed that the unit employed delivered 1.55 ml. per stroke, the vaccine to be used was appropriately diluted so that each stroke was equivalent to 1.0 ml. of the original preparation.

Six ferrets were individually sprayed in conventional mink nest boxes, the openings of which were sealed with cardboard save for a small spraying hole. Spraying was at the rate of one stroke per nest box, following which the spraying holes were taped and the animals kept confined for 15 minutes before being removed to their cages. Two normal ferrets were then placed with the sprayed animals to serve as contact controls, and 2 others were injected intraperitoneally, each with 1.0 ml. of the original vaccine. All 8 animals and 2 additional normal controls were challenged 14 days later. Only 3 of 6 sprayed animals were immune (table 4). The other

* DeVilbiss Co.

† Z and W Machine Products Co.

TABLE 3—Potency Testing of Distemper Vaccine Lot 6368-30A

Group No.	No. of ferrets	Method of vaccination	Interval between exposure and challenge (days)	Results of challenge
1	6	Reconstituted vaccine, spray	13	Survived 21 days
2	2	Reconstituted vaccine, intraperitoneally	13	Survived 21 days
2a	2	None	Controls for groups 1 and 2	Died 13 and 15 days

TABLE 4—Application of "Atomized" Aqueous Vaccine by Z and W Sprayer

Group No.	No. of ferrets	Method of vaccination	Interval between exposure and challenge (days)	Results of challenge
1	6	Reconstituted vaccine, spray	14	3 died in 12, 14, and 24 days
1a	2	None	Contacts with group 1	Died 12 and 12 days
2	2	Reconstituted vaccine, intraperitoneally	14	Survived 30 days
2a	2	None	Controls for groups 1 and 2	Died 14 and 14 days

3, as well as the contact and challenge control ferrets, became sick and died, whereas those receiving the vaccine intraperitoneally survived challenge.

EXPERIMENT IN MINK

Evaluation of the efficacy of distemper vaccine in mink is rendered somewhat difficult by the fact that only a proportion of reportedly susceptible animals die of the disease when inoculated with a fully virulent strain of virus. For example, in one experiment, Gorham *et al.*[5] inoculated 25 unvaccinated mink with virulent virus. Of these, 18 showed signs of infection and only 10 died of the disease. In later publications,[6,7] the same group of investigators abandoned the challenge method and resorted to the measurement of neutralizing antibodies following the experimental vaccination of mink.

One of us [14] has found over the past several years that the CF method is an effective tool for assessing the response of dogs to vaccination with modified distemper virus of avian origin. Continuous use of this method has shown that 100 per cent of susceptible pups give positive CF titers two to three weeks following a single injection of vaccine by either the subcutaneous, intramuscular, or intradermal routes. The success of the CF method in dogs suggested its use in the following experiment in mink.

Blood specimens were taken by heart puncture from normal mink and their serum saved for future testing by the CF method. Since the results obtained in ferrets with the Z and W sprayer were poorer than those produced by the DeVilbiss 40 nebulizer, 21 animals were sprayed with the latter in individual nest boxes at the rate of 1 ml. of vaccine per mink, under about 8 lb. water-pumped nitrogen pressure. It took about 70 seconds at that pressure to nebulize 1 ml. of vaccine. To avoid excessive leaks, the nest boxes were covered with canvas at the time of spraying, and the nose of the sprayer was inserted under the canvas. After delivery of the vaccine, the canvas was left on for approximately ten to 15 minutes. Two additional mink were each injected subcutaneously with 1 ml. of the vaccine and 3 normal animals were left untreated to serve as controls.

Within two days after vaccination, 3 of the sprayed animals and 1 of the subcutaneous group died, probably as a result of heart puncture or heat prostration. The remaining mink were bled again 30 days after exposure to the vaccine, and both their pre- and postvaccination serums were tested for CF antibodies.

The results (table 5) show that of the 18 sprayed mink, only 1 had a positive titer before vaccination, while the number of positive serums rose to 11 after vaccination. If the one specimen found positive before and after spraying is excluded from calculation, 59 per cent of the mink acquired CF antibodies. The surviving animal of the subcutaneous group also

TABLE 5—Distemper Vaccination of Mink with "Atomized" Aqueous Vaccine

Group No.	No. of mink	Method of vaccination	Complement-fixation results: Prevaccination titers		Postvaccination titers * 30 days after exposure					
			Neg.	1:8	Neg.	1:8	1:16	1:32	1:64	1:128
1	18	Reconstituted vaccine, spray	17	1	7	3	—	3	2	3
2	1	Reconstituted vaccine, subcutaneous	1	—	—	—	1	—	—	—
3	3	None, controls	3	—	3	—	—	—	—	—

* Discounting the animal with a positive complement-fixation (CF) titer on the prevaccination sample, 59 per cent of sprayed mink developed CF antibodies as a result of spraying.

became positive as expected, whereas control mink remained negative.

DISCUSSION

Under experimental conditions designed to simulate those prevailing in the field, distemper vaccination of ferrets with atomized vaccine was possible when the vaccine was applied in an aqueous rather than a dust form. The failure to immunize with dust contrasts with the success of the method in chickens but may be explained, at least in part, by the differences between the nasal passages of chickens and ferrets. Furthermore, the conjunctiva also serves as a portal of entry for Newcastle disease and infectious bronchitis virus vaccines, but it has not been found possible to immunize young pups with avian distemper virus by that route.[14] The successful results reported by others[7] with a dust vaccine were obtained by holding the head of the animal tightly against the orifice of a glass cylinder directly connected to the sprayer, thus "forcing" the virus down the respiratory system.

The size of the mist particle is to be given serious consideration when an atomized aqueous vaccine is applied. While the DeVilbiss 40 nebulizer was found 100 per cent effective in every ferret experiment, the Z and W sprayer, which delivers much coarser particles, protected only 50 per cent of the animals. The fog produced by the former could be seen suspended for several minutes, but the spray of the latter followed a rapidly descending trajectory.

The CF method seems to be of as much value in measuring the immune response of mink to distemper vaccination as it was found to be in dogs. The seemingly inferior results obtained above may have been due to several factors. The time of the postvaccination bleeding may not have been optimum, for example. Furthermore, the canvas cover did not supply a tight closure, and the nebulizer had to be held at an appreciable angle under one corner of the canvas, precluding optimal spraying conditions. Furthermore, there were holes in the partitions dividing the nest boxes used in the experiment, so an animal could have hidden in a corner remote from the spray.

SUMMARY

An "atomized" dust distemper vaccine of avian origin failed to protect ferrets under conditions similar to those which would be used in the field. In contrast, an atomized aqueous vaccine was repeatedly found successful. The success of this latter method, however, depends largely on the size of the mist particle: a fine nebulizer consistently afforded 100 per cent protection, a coarser sprayer immunized only 50 per cent of the animals.

The complement-fixation (CF) method was found to be a valuable tool for measuring the immune response of mink to the same atomized aqueous distemper vaccine: 59 per cent of animals vaccinated by that method under somewhat adverse conditions developed distemper CF antibodies.

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Diseases Seen at Necropsy of 708 *Macaca Mulatta* (Rhesus Monkey) and *Macaca Philippinensis* (Cynomolgus Monkey)

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THIS STUDY is based upon the postmortem findings in 708 young monkeys which were obtained by the National Institutes of Health from several importers. In some shipments of monkeys, there were high rates of sickness and death, and examinations were made on the moribund and dead animals. The main losses in the animals appeared to be due to tuberculosis, pneumonia, salmonellosis, shigellosis, gastrointestinal parasites, and malnutrition.

In 1950, the occurrence of different diseases of monkeys was little known. Workers were concerned about the usefulness of diseased monkeys in exposure studies of infectious diseases especially when changes might possibly be due to ascribed or intercurrent processes. When the naturally occurring conditions were known and evaluated, preventive measures and treatments to eliminate the diseases from monkeys could then be studied.

The purpose of this study was to determine the percentage of occurrence of the bacterial, viral, parasitic, neoplastic, and nutritional diseases, and to study the histopathological changes produced by the naturally occurring diseases of monkeys.

MATERIALS AND METHODS

During the period, July 26, 1950, through Sept. 8, 1955, necropsies were done on 615 rhesus and 93 cynomolgus monkeys—378 males and 330 females. Most of the animals were young, weighing from 2 to 6 lb. The animals were brought to the National Institutes of Health in shipments of 25 to 100. During the first four years of this study, the monkeys were kept in large open cages with cement floors, tile walls, and outside runs. Each cage would accommodate from 35 to 50 monkeys. Later, June, 1954, the monkeys were kept in small portable cages, each housing 2 rhesus or 2 cynomolgus monkeys. The animals were held in quarantine from one to eight weeks.

At the beginning of this study, the animals were fed a grain diet with a liberal quantity of fruit. Later, in June, 1954, the monkeys were fed baked biscuits with oranges for vitamin C supplement three times a week. The animals obtained their drinking water from an open fountain at first but, later, when they were kept in small cages, they were offered water from 1,000-cc. water bottles.

All the monkeys were given the intrapalpebral tuberculin test by the injection of approximately 0.1 cc. of tuberculin within one week after their arrival. The tuberculin tests were repeated every four to six weeks, and all the reactors were killed.

Necropsies were performed on all moribund and dead animals. Dead animals were placed in a refrigerator, and the gross lesions were noted in each necropsy. Bacteriological cultures were made from diseased tissues from 370 rhesus and 60 cynomolgus. Tissues from these monkeys were also saved for histological study.

Fecal samples for examination for gastrointestinal parasites, by use of the salt flotation-centrifugation technique, were obtained from each of the monkeys, except those that had tuberculosis or were positive to the tuberculin tests. No fecal examinations were made for protozoa.

Bacteriological examinations of fecal material for the isolation of Salmonella spp. were done by inoculating tubes of cystine selenite F. broth with about 1 Gm. of fecal material and, after an incubation period of 18 to 24 hours, the broth cultures were streaked on SS agar plates. After an incubation period of 18 to 24 hours, suspicious colonies were picked from the SS agar plates. Shigella spp. were isolated from fecal material and scrapings of the upper colon. The differential mediums and procedures used for the isolation of Salmonella and Shigella spp. were the same as those described by Galton *et al.*[1] All Salmonella and Shigella cultures were typed for species.*

From June, 1955, antibiotics were administered to the sick monkeys. Penicillin was administered to monkeys with pneumonia and chloromycetin was offered in the drinking water to monkeys with gastroenteritis.

RESULTS

The pertinent results and the occurrence of tuberculosis in the 615 rhesus monkeys is shown (table 1); 91 had tuberculosis of

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The writers express their appreciation to Dr. George L. Fite for his help on the histopathology and his advice in this study.

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the hilar lymph nodes, lungs, and other organs and 10 additional monkeys showed generalized tuberculosis by gross examination; thus, 101 of the rhesus monkeys (16.4 %) had proved tuberculosis. In addition, 31 monkeys (5.0 %) reacted to the tuberculin test, but showed no tuberculous lesions on gross examinations, and no acid-fast bacilli were seen in suspicious lesions on microscopic examination. These unconfirmed cases of tuberculosis were probably active cases, but the foci of infection were so small that they were not found. The total number of cases of tuberculosis, therefore, was 132 (21.5 %).

No determinations of the accuracy of the palpebral tuberculin tests were made because in the early part of the study many tuberculous monkeys died soon after their arrival before the tuberculin test could be performed. However, Habel [2] has shown that there is a high degree of correlation between the tuberculin test and necropsy evidence of tuberculosis.

Ninety-three cynomolgus monkeys were tuberculin tested and examined for gross

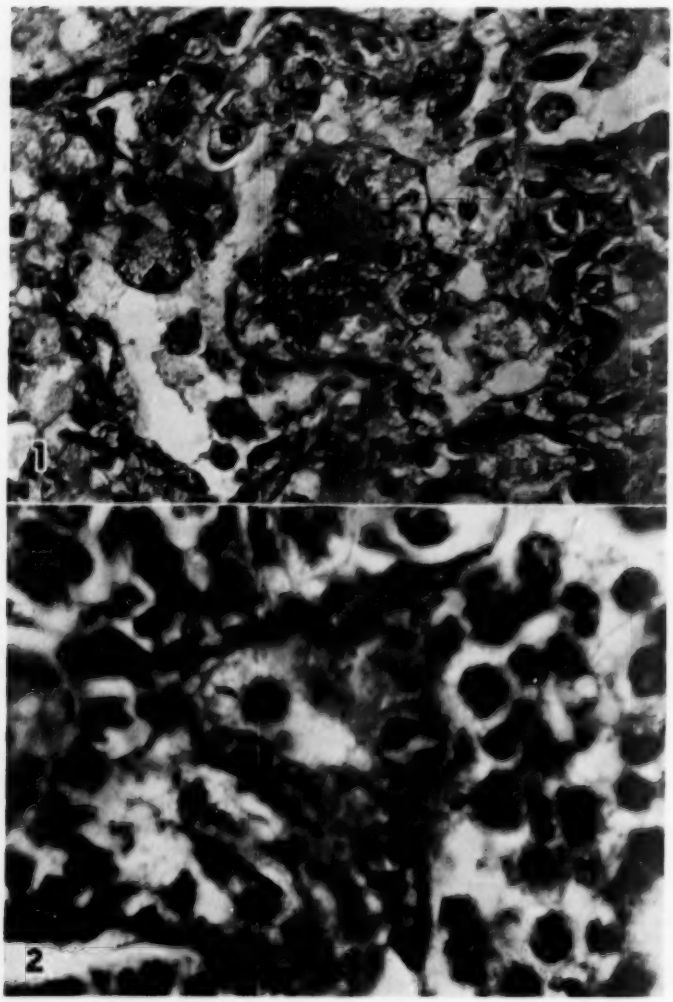

Fig. 1—Alveoli with a multinucleated giant cell containing numerous intranuclear inclusion bodies. x 517.

Fig. 2—Purulent exudate from within bronchiole, large cell with prominent intranuclear and variously sized cytoplasmic inclusion bodies, and numerous cytoplasmic inclusions in epithelial cells. x 1,000.

TABLE 1—The Occurrence of Tuberculosis Among 615 Deaths in Rhesus Monkeys

	Microscopic examinations		Gross examinations		Reactors to the test			
	No. of animals	Infected (%)	No. of animals	Infected (%)	No. of animals	Infected (%)	Total infected	Infected (%)
Tuberculosis of lung or hilar lymph nodes	52	8.5	7	1.1	*	....	59	9.6
Generalized tuberculosis	39	6.3	3	0.5	*	....	42	6.8
No lesion reactors	....	....	....	....	31	5.0	31	5.0
Total	91	14.8	10	1.6	31	5.0	132	21.5

* Tuberculin test not administered.

lesions and the tissues of 60 of these were examined microscopically for lesions. No evidence of active tuberculosis was found, although 3 animals were classed as reactors to the tuberculin test. Inasmuch as the cynomolgus monkeys were kept in the same rooms, were given the same routine care, and were probably exposed to tubercule bacilli as much as the rhesus monkeys, it appears that the cynomolgus monkeys are considerably more resistant to tuberculosis than the rhesus monkeys.

Bacterial pneumonia was the principal cause of death of 113 (15.8 %) of 708 monkeys. This disease was responsible for the death of 86 of 615 rhesus monkeys (14.0 %) and of 27 of 93 cynomolgus monkeys (29.0 %). Interstitial pneumonia was the cause of three deaths (0.5 %) of the rhesus and four deaths (4.3 %) of the cynomolgus monkeys.

A giant cell pneumonia was observed in 2 cynomolgus monkeys but was not seen in the rhesus monkeys. This giant cell pneumonia with intranuclear and cytoplasmic inclusion bodies was first described by Pinkerton *et al.*[3] as a distinctive disease of infancy and enildhood. This pneumonia is characterized, histopathologically, as an interstitial pneumonia in which frequent, large, multinucleated, foreign-body type giant cells with large eosinophilic intranuclear inclusion bodies are seen in the distended alveoli (fig. 1). Intranuclear and cytoplasmic inclusion bodies were also seen in the alveolar and bronchial epithelial cells. Some of these epithelial cells increase in size, bud off of the mucosa, and have large intranuclear and many smaller cytoplasmic inclusion bodies (fig. 2). This disease may occur more frequently in both groups of monkeys and probably has been overlooked in some of the animals, since it has been noted that after secondary infections become prevalent the tissue pathology of the lung changes to one of bacterial pneumonitis.

Enteritis was diagnosed in 291 (47.3 %) of 615 rhesus monkeys and was the attributed cause of death in 50 (53.8 %) of 93 cynomolgus monkeys. The precise cause of enteritis in this latter group was not determined but, when bacterial tests for Salmonella and Shigella spp. were negative and the numbers of helminths in the animals were considered not numerous enough to cause death in the monkeys, this condition was attributed to inflammation of the intestinal tract.

The results of the bacteriological examinations of feces for the isolation of Salmonella and Shigella spp. are shown (table 2). It should be pointed out that, in group 1, bacterial isolations were made from feces collected from untreated monkeys and, from group 2, isolations were made

TABLE 2—The Relationship of Medication to Isolations of Salmonella and Shigella Spp. in 275 Rhesus Monkeys

	Salmonellosis		Shigellosis		Totals		
	No. of animals	Animals infected (%)	No. of animals	Animals infected (%)	No. of animals	No. of animals infected	Animals infected (%)
Group 1 untreated	27	19.5	10	7.2	138	37	26.9
Group 2 treated	14	10.2	18	13.1	137	32	23.3
Total	41	14.9	28	10.2	275	69	25.1

from feces of medicated monkeys. However, many of the monkeys in the treated group were sick and weak when treated and many died before any beneficial effects could have been derived from the medication. Salmonella spp. were isolated from 27 monkeys in group 1 (138 animals), and from 14 monkeys in group 2 (137 animals). The number of isolations of Salmonella spp. from both groups (275 animals) was 41 (14.9 %). Shigella spp. were isolated from 10 rhesus monkeys in group 1, from 18 rhesus monkeys from group 2, and from both groups from 28 monkeys or a percentage isolation of Shigella spp. of 10.2 per cent.

TABLE 3—Occurrence of Helminth Eggs in 530 Rhesus and 82 Cynomolgus Monkeys

Infection	Rhesus		Cynomolgus	
	No. of animals	Percentage of animals	No. of animals	Percentage of animals
Ascariasis	3	0.6	0	0
Taeniasis	3	0.6	0	0
Strongyloidiasis	129	24.3	6	7.3
Oesophagostomiasis	71	13.4	4	4.9

Fecal samples were collected at necropsy from 23 cynomolgus monkeys for the isolation of Salmonella and Shigella spp. *Salmonella enteritidis* was recovered from 1 (4.3 %) and no isolations of Shigella spp. were made in any of the monkeys.

Paracolon bacilli were isolated from 7 rhesus monkeys, and Bethesda paracolon was the species isolated from four of the seven isolations.

The number of rhesus monkeys and the different Salmonella spp. that were isolated from 41 infected monkeys were as follows: *S. enteritidis*, 14; *Salmonella typhimurium*, 16; *Salmonella stanley*, 5; *Salmonella san diego*, 2; *Salmonella anatum*, 2; *Salmonella barcilly*, 1; and *Salmonella braenderup*, 1.

The number of monkeys and the different Shigella spp. isolated from 28 infected rhesus monkeys were as follows: *Shigella flexneri* 2a, 15; Shigella 4a, 6; Shigella 4b, 5; Shigella 6b, 1; Shigella 4a and 4b, 1; and Shigella serotype 123-1.

The results of the salt-flotation fecal examinations from 530 rhesus and 82 cynomolgus monkeys are shown (table 3). As carids and tapeworms, *Bertiella studeri*, were present in 3 of the rhesus monkeys but neither were found in the cynomolgus monkeys. Strongyloides sp. eggs and Oesophagostomum spp. eggs occurred frequently in the fecal specimens of the rhesus monkeys, but less frequently in the cynomolgus monkeys. Parasitisms due to the above parasites was diagnosed as the cause of death in 17 (3.2 %) rhesus and 3 (3.7 %) cynomolgus monkeys.

Four nematodes,† Trichuris spp., *Streptopharagus armatus*, *Paraoesophagostomum polydentatum*, and *Turnidens deminutus*, were recovered from the cecums of 4 rhesus monkeys.

The larvae of Oesophagostomum spp. produced abscesses in the submucosa and serosa of the cecum and colon in 12 (3.2 %) of 370 rhesus monkeys (fig. 3). Microscopically, the lesions showed segments of the nematodes surrounded by an infiltration of polymorphonuclear leukocytes, eosinophils, plasma cells, and lymphocytes and the periphery encapsulated by a dense band of fibrous connective tissue. In some instances, a fistulous tract was seen leading from the lesion in the submucosa to the lumen of the colon and, in older lesions, fibrous granulomas were seen with deposition of calcium around the parasite and in the capsule.

Fig. 3—An abscess containing segments of nematode larvae in submucosa of colon. x 33.

† Identifications of the species of helminths were made by Allen McIntosh of the Animal Disease and Parasite Research Branch, Beltsville, Md.

The other parasites that were found on microscopic examination of tissues from 370 rhesus and 60 cynomolgus monkeys were flukes, capillarids, Balantidium, Sarcosporidia, and lung mites. Liver flukes were found in the bile ducts of 1 rhesus monkey. Identification of these flukes was not made, and liver flukes, *Clonorchis sinensis*, were seen in 3 cynomolgus monkeys (fig. 4). *Capillaria hepatica* eggs were seen in the liver in 1 rhesus monkey (fig. 5). Balantidium spp. were seen in the lu-

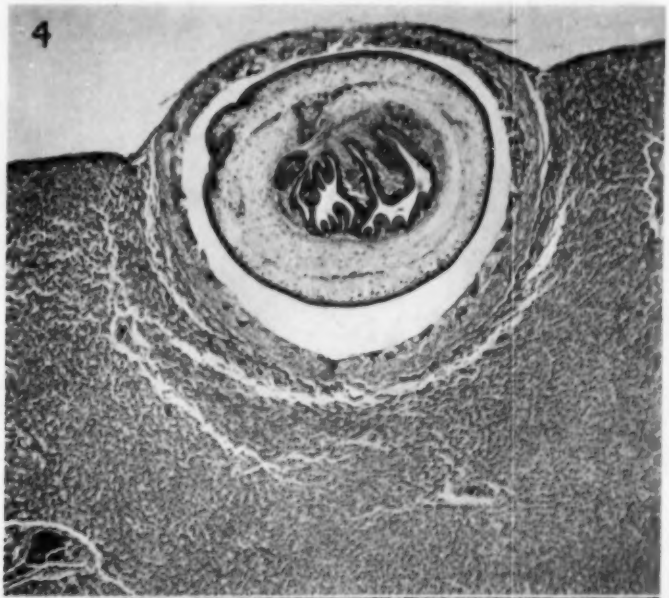

Fig. 4—Segment of Clonorchis sinensis in dilated bile duct of cynomolgus monkey. x 55.

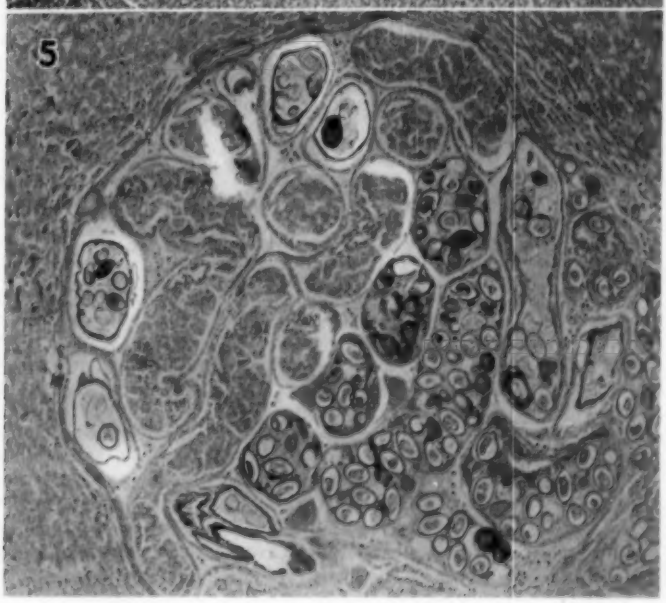

Fig. 5—Encapsulated segments of capillarids and masses of Capillaria hepatica eggs in the liver of rhesus monkey. x 100.

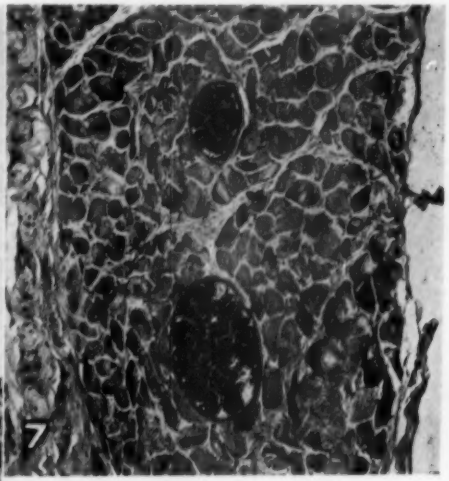

Fig. 6—Lung mites (arrows), Pneumonyssus semicola, in a bronchiole. x 90.

Fig. 7—Sarcosporidia in skeletal muscle of rhesus monkey. x 120.

men and in the crypts of the large intestines in 5 rhesus monkeys. This protozoan parasite is a common intestinal parasite of man in Europe [4] but rarely found in man in the United States. Lung mites, *Pneumonyssus semicola*, were seen in bronchioles and subpleural cystic areas in 103 rhesus monkeys (fig. 6). The prevalence and pathological changes produced by lung mites has been described by Innes *et al.*[5]

Sarcocystis kortei were seen in the skeletal muscles of 4 rhesus monkeys (fig. 7). Although Sarcosporidia are occasionally seen in the muscles of the neck,[6] heart, and in striated muscles of monkeys, it is rarely seen in man.[7]

Calcareous concretions, corpora calcificans, identical to those described by Ross *et al.*[8] in the corticomedullary region of the adrenals were seen in 23 (6.2 %) of the rhesus monkeys.

At necropsy, of the 615 rhesus monkeys, 45 (7.3 %) were completely autolyzed and, of the 93 cynomolgus monkeys, 9 (9.8 %) were autolyzed or undiagnosed cases.

No neoplasms were seen among the 370 rhesus and 60 cynomolgus monkeys examined histologically; however, most of the monkeys examined were young and could not be considered in the cancer age group.

DISCUSSION

The main cause of death in the rhesus and cynomolgus monkeys was often difficult to determine because two or more diseases usually occurred concurrently.

Enteritis produced the highest number of deaths in the monkeys, but the exact cause of the enteritis was not determined in every case. Early in the study, it was apparent in new shipments of young monkeys that much of the diarrhea was due to abrupt changes in diet, inadequate diet, exposure, and to shipping sickness. Ratcliffe [9] and Jukes and Stockstad [10] reported that monkeys were frequently fed protein-deficient diets, and that when these monkeys were fed adequate diets, their diarrhea, due to bacillary and amebic dysentery, disappeared.

Hardy [11] reported that a high incidence of diarrhea occurred in a group of monkeys that had heavy infections of Salmonella and Shigella spp. and a low incidence in a group from which few of these two groups of organisms were isolated. He also reported that the number of cases of enteritis in monkeys varied considerably

in different shipments and that this condition was more serious during the hot months of the year.

Barnes *et al.*[12] isolated eight types and subtypes of Shigella spp. from 346 monkeys and found 52.9 per cent of the animals infected. It is also probable that paracolon and coliform organisms may have pathogenic properties and produce enteritis. Moreover, different viruses could be the initial causes of enteritis in some of the animals but be unobserved because of secondary invaders. However, there was no histopathological evidence that viruses were solely responsible for any of the cases. Intestinal parasites were undoubtedly responsible for the diarrhea and enteritis in many of the animals, especially when large numbers of protozoa, larvae, or mature worms were present.

Pneumonia was responsible for a high mortality in the monkeys and occurred concurrently with other diseases. Lobar and bacterial pneumonia was the usual type, except for 7 cases of interstitial pneumonia and 2 cases of giant cell pneumonia. Giant cell pneumonia may be a serious cause of pneumonia in rhesus and cynomolgus monkeys and may be a source of infection for man.

Lung mites were frequently seen in the lungs of young monkeys, but they produced localized cystic lesions and did not cause extensive pneumonia.

Tuberculosis was the cause of many deaths in rhesus monkeys. It was not diagnosed in cynomolgus monkeys by microscopic examination of tissues. Three of these animals were reactors to the tuberculin test.

No histopathological evidence or specific lesions attributable to malnutrition and vitamin deficiencies were seen.

Summary and Conclusions

1) The gross and microscopic findings and the percentage occurrence of diseases among necropsies performed on 615 rhesus and 93 cynomolgus monkeys are presented.

2) The principal causes of death in 615 rhesus monkeys were: enteritis, 47.3 per cent; tuberculosis, 21.5 per cent; pneumonia, 14.0 per cent; and parasitism, 3.2 per cent.

3) The principal causes of death in 93 cynomolgus monkeys were: enteritis, 53.8 per cent; pneumonia, 29.0 per cent; tuberculosis, 0 (3 reactors to tuberculin test); and parasitism, 3.7 per cent.

4) The number of isolations of Salmonella and Shigella spp. from 275 rhesus monkey fecal samples was 14.9 and 10.2 per cent, respectively.

5) *Salmonella enteritidis* was isolated from 1 of 28 cynomolgus fecal samples examined for Salmonella and Shigella spp.

6) The species of Salmonella isolated from 41 infected rhesus monkeys were: *Salmonella typhimurium*, 16; *S. enteritidis*, 14; *Salmonella stanley*, 5; *Salmonella san diego*, 2; *Salmonella anatum*, 2; *Salmonella bareilly*, 1; and *Salmonella braenderup*, 1.

7) The species of Shigella isolated from 28 infected rhesus monkeys were: *Shigella flexneri* 2a, 15; Shigella 4a, 6; Shigella 4b, 5; Shigella 6b, 1; Shigella 4a and 4b, 1; and Shigella serotype 123-1.

8) The kinds of internal parasites recovered and the number of positive nematode egg fecal samples obtained from 530 necropsied rhesus monkeys were ascarids and tapeworms, 0.6 per cent; Strongyloides sp., 24.3 per cent; and Oesophagostomum spp., 13.4 per cent.

9) The number of cynomolgus monkeys infested with Strongyloides sp. and Oesophagostomum spp. from fecal examinations from 82 monkeys were 7.3 and 4.9 per cent, respectively.

10) Microscopic abscesses of the submucosa and serosa of the cecum and colon caused by larvae of Oesophagostomum spp. were found in 12 of 370 rhesus monkeys.

11) Other parasites that were found on microscopic examinations of tissues from 370 rhesus monkeys were *Pneumonyssus semicola*, liver fluke not identified, *Capillaria hepatica*, Balantidium spp., and *Sarcocystis kortei*.

12) *Clonorchis sinensis* was the only parasite found in the microscopic examination of tissues from 60 cynomolgus monkeys.

13) Giant cell pneumonia with intranuclear and cytoplasmic inclusion bodies was seen in 2 cynomolgus monkeys.

14) Corpora calcificans were seen in the adrenals of 6.2 per cent of the rhesus monkeys.

15) No neoplasms were observed at nec-

ropsy in any rhesus or cynomolgus monkeys.

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Preliminary Investigation into the Life Cycle of the Monkey Lung Mite (*Pneumonyssus Foxi*)

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DURING the course of some 600 necropsies of *Macaca mulatta* monkeys imported from India,[1] pulmonary infection with the lung mite, *Pneumonyssus foxi*, was observed without exception. Fairbrother and Hurst[2] reported an incidence of approximately 50 per cent in a group of 597 *Macaca rhesus* monkeys; Helwig[3] reported 6 of 9 monkeys infected with *P. foxi* in 1925; and Gay[*] and Branch[4] reported acariasis in 20 of 25 *M. rhesus* monkeys in 1926. These high incidences of infection led to speculation that some unusual mode of transfer might occur in the life cycle of this mite. Disclosure at the 1954 course, Pathology of Diseases of Laboratory Animals,[*] and by Lee *et al.*[5] that the lung mite is capable of penetrating into the blood stream of monkeys, led to the conclusion that transplacental transfer of this mite during its life cycle should be proved or disproved.

This report concerns an investigation to establish whether transplacental transfer of the lung mite, *P. foxi*, occurs in the *M. mulatta* monkey.

MATERIALS AND METHODS

It was assumed that the entire laboratory facility housing *M. mulatta* monkeys at the Radiobiological Laboratory, as well as the residences of the investigators, was contaminated by the lung mite. It was, therefore, deemed necessary to deliver some of the fetuses under sterile conditions by cesarotomy at a site remote from the laboratory and then to transfer the infant monkeys to a third remote site for raising. Cesarotomies were done on 5 monkeys, at term, at five different veterinary hospitals and the infant monkeys were transferred to a sixth veterinary hospital.

The young monkeys were killed for necropsy at the following postdelivery periods: 1 at one hour, 2 at ten days, 1 at 15 days, and 1 at 31 days; also an infant monkey, born naturally and maintained in intimate contact with her parents, at 306 days of age. The lungs of 2 9-month-old *M. mulatta* monkeys raised in an Ohio colony † were contributed for study.

The lungs were fixed in 10 per cent formalin immediately following death, with the exception of those contributed which were profused with Zenker-formalin solution and stored in 70 per cent alcohol for nearly three years prior to examination. The fixed lungs were sectioned serially in 10-μ sections with every thirty-fifth section removed for hematoxylin-eosin staining and detailed microscopic examination.

RESULTS

No evidence of *P. foxi* infection was found on detailed microscopic examination of hematoxylin-eosin stained serial sections of the lungs of 8 immature *M. mulatta* monkeys.

CONCLUSIONS

This study indicates that transplacental transfer is unlikely to occur in the life cycle of *Pneumonyssus foxi*. Further, the environmental conditions at Christ Hospital and the Radiobiological Laboratory appear to be unfavorable for the transfer of lung mites from adult *Macaca mulatta* monkeys to immature monkeys up to the age of 306 days.

The raising of *M. mulatta* monkeys free of lung mites for research purposes can apparently be accomplished without diffi-

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Deep appreciation is expressed by the authors to Dr. and Mrs. D. D. Linam who raised the infant monkeys and to Drs. H. M. Spangler, G. D. Stallworth, Henry Tischler, O. F. Goen, D. A. Darter, and N. L. McLeod for assistance and provision of facilities for the cesarean sections.

This project was supported in part by contract AF 18(600)-165 and the School of Aviation Medicine.

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† By Dr. L. H. Schmidt of Christ Hospital, Cincinnati, Ohio.

culty in the environments considered by this study even though the life cycle of *P. foxi* remains an enigma.

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An Evaluation of the Efficacy of Stylomycin Phenothiazine, Cadmium Anthranilate, and the Piperazine Compounds for the Removal of Oxyurids in Mice

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THIS STUDY was initiated in an attempt to determine the least toxic and most effective medicament that may be administered in a single treatment in the feed for the removal of *Aspicularis tetraptera* and *Syphacia obvelata* from mice.

Habermann and Williams [4] reported that phenothiazine alone was more effective than chlortetracycline, bacitracin, and oxytetracycline when these antibiotics were added to phenothiazine for the removal of oxyurids from mice.

An anthelmintic that is less toxic than phenothiazine that will effectively remove the oxyurids in a single treatment when offered in the feed and eliminate 95 per cent or more of the oxyurids in mice is needed. Such a drug is also needed for the removal of *Enterobius vermicularis* in man and for the nematodes of domestic and laboratory animals.

Standen [6] found, in screening a large number of anthelmintic compounds, that gentian violet and piperazine hydrate were the only effective drugs in the removal of oxyurids from mice. Brown *et al.*[2] observed, in screening a group of 32 piperazine compounds against *S. obvelata* in mice, that the most effective drug tested was the single piperazine base and its salts.

Bradley *et al.*[1] found that piperazine citrate was 100 per cent effective for the removal of ascarids and 42.8 per cent effective for the removal of *Trichuris vulpis* from dogs. Kelley *et al.*[5] ran field trials on the efficacy of piperazine adipate,* parvex,Ⓟ† and cadmium anthranilate‡ in the treatment of ascariasis in swine. They found that piperazine adipate and parvex reduced the egg count 99 per cent in eight days, while cadmium anthranilate reduced the egg count 98 per cent in 21 days.

Grumble *et al.*[3] found that stylomycin § was less effective than piperazine hexahydrate against *A. tetraptera* but more effective against *S. obvelata* in a single oral dose.

MATERIAL AND METHODS

In this study, 370, 1-month-old, female, white Swiss mice, weighing 10 to 12 Gm., were used. Experimental infections of *A. tetraptera* and *S. obvelata* were obtained in the mice by placing 45 to 50 mice in an infection cage containing 10 to 12 heavily parasitized animals, according to the technique of Habermann and Williams.[4] At the termination of the exposure period, experimental mice were placed in three to five groups (10 animals per group) by random selection and offered a medicated feed prepared by mixing the drug with 4 to 5 ml. of molasses which, in turn, was then admixed with 100 Gm. of ground Purina mouse feed. Control groups received 100 Gm. of ground Purina feed (10 Gm./mouse) mixed with 4 to 5 ml. of molasses.

Stylomycin was offered at the rate of 3 mg./10 Gm. of molasses feed (300 mg./kg. of body weight). Animals treated with the aminonucleoside of stylomycin were offered the drug at the rate of 1 mg. and 3 mg./10 Gm. of molasses feed. Phenothiazine (N.F. green) was offered to mice at the rate of 500 mg./10 Gm. of feed. Mice treated with piperazine adipate and pipcide ‖ received 5 mg. and 10 mg./10 Gm. of feed. Parvex was offered to mice at the rate of 10 and 20 mg./10 Gm. of feed. Mice treated with cadmium anthranilate were offered the drug at the rate of 25 and 30 mg./10 Gm. of feed. Each control mouse was offered 10 Gm. of unmedicated molasses feed.

At the end of the 24-hour period, the remaining portion of the medicated feed was moistened with a small amount of tap water and loosened from the sides of the feeding jars. The mice consumed all of the feed within a 48-hour period.

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* The adipic acid derivative of piperazine (Carson Chemicals, Inc.).

† Piperazine-carbon disulfide complex (betaine of 1-piperazine carbodithioc acid) in an inert carrier (Upjohn Co.).

‡ Sold under the name "cadmium hog wormer," containing 4.4 per cent anthranilate (Clark and Hess, Inc.).

§ Puromycin HCl, 6-dimethylamino-9-(3'-p-methoxy-L-phenyl-alanylamino-3'-deoxy-D-riboxyl-purin (American Cyanamid Co.).

‖ The piperazine citrate and piperazine tartrate complex in equimolecular amounts (Haver-Glover Laboratories).

TABLE 1—Efficacies of Seven Anthelmintics for the Removal of Pinworms from Mice

Drug	Dosage (mg./10 Gm. of feed)	No. of animals	No. worms passed	No. worms recovered		Total recovered	Total worms	Av. efficacy (%)
				Cecum	Colon			
Stylomycin	3	90	4,692	53	27	80	4,772	98.1
Phenothiazine	50	50	1,768	505	247	752	2,520	70.1
Pipcide	5 to 10	30	344	1,375	1,214	2,589	2,933	11.7
Parvex	10 to 20	30	944	1,071	128	1,199	2,143	44.04
Piperazine adipate	10	30	999	569	413	982	1,981	50.4
Aminonucleoside of stylomycin	1 to 3	20	764	744	197	941	1,705	44.8
Cadmium anthranilate	25 to 30	30	876	532	175	707	1,583	55.3
Controls	....	90	1,130	5,119	2,369	7,488	8,618	13.1

Following the first day of treatment, fecal material was collected from each group for two consecutive days, screened in 20- and 60-gauge wire pans, and the total number of worms passed was counted.

On the third day following treatment, necropsies were conducted on each mouse, cecum and colon contents were screened, and the total number of worms recovered was recorded. Gross necropsy findings were recorded, tissues were saved for histological examination, and the drug efficacy of each anthelmintic was determined.

Results

The dosage, anthelmintics tested, number of worms passed, number of worms recovered from the cecum and colon at necropsy, and the percentage of efficacy against oxyurids in mice is shown (table 1).

Stylomycin was the most effective drug, 98.1 per cent of the worms being eliminated following its use. In 90 mice treated with this antibiotic, only 53 worms were recovered from the cecum and 27 from the colon. There was no evidence of toxicity and no abnormal effects as a result of the treatment.

Phenothiazine was effective in the removal of 70.1 per cent of the oxyurids. From 50 treated mice, 505 oxyurids were recovered from the cecum as compared to 247 from the colon.

Cadmium anthranilate was effective in the removal of 55.3 per cent of the worms; from 30 mice, 532 oxyurids were recovered from the cecum and 175 from the colon.

Piperazine adipate-treated mice eliminated 50.4 per cent of the worms; 569 oxyurids were recovered from the cecum and 413 from the colon.

In two groups of 39 mice treated with pipcide and parvex, eliminations of 11.7 per cent and 44.04 per cent of the worms were obtained, respectively. A total of 1,375 oxyurids were recovered from the cecum and 1,214 from the colon in the pipcide-treated mice, and the parvex group harbored 1,071 oxyurids in the cecum and 128 oxyurids in the colon at necropsy. The elimination of worms from the colon in the latter group showed a significant increase as compared with other experimental groups (table 1).

Only 44.8 per cent of the worms were eliminated by mice treated with aminonucleoside of stylomycin. Although poor drug efficacy was obtained, there was a decrease in worm burden from the colons of these animals. In a group of 30 mice, a total of 744 oxyurids were recovered from the cecum and 175 from the colon.

In the untreated control group, only 13.1 per cent of the worms were removed spontaneously. A total of 5,119 oxyurids were recovered from the cecum and 2,369 from the colon.

Gross and microscopic examination of viscera from animals in all groups showed no evidence of drug toxicity. Throughout the experiment, both the treated and untreated mice ate normally, appeared active, and had smooth, glossy coats. Tapeworm segments were observed in the small intestines of 2 mice receiving stylomycin.

Discussion

In this study, the elimination of 100 per cent of the oxyurids occurred in one group of mice treated with stylomycin. However, Grumble *et al.*[3] reported that piperazine hexahydrate was more effective than stylomycin in the removal of *A. tetraptera* from mice in a single dose. Their results were probably due to the fact that their mice were not as heavily parasitized as the experimentally infected mice used in the present study.

Piperazine compounds in the present study may have been given in insufficient concentrations to effectively remove the oxyurids of mice in a single treat-

ment. A treatment study of this type will be done at a later date.

CONCLUSIONS

1) Stylomycin was nontoxic and was the most effective of the anthelmintics tested for the removal of *Aspicularis tetraptera* from the colon and *Syphacia obvelata* from the cecum of mice, eliminating 98.1 per cent of the pinworms (uncorrected average efficacy).

2) Phenothiazine was 70.1 per cent effective for the removal of worms from the mice.

3) Parvex,® pipcide, piperazine adipate, aminonucleoside of stylomycin, and cadmium anthranilate were not as effective as stylomycin and phenothiazine in the elimination of oxyurids from mice, expelling only 44.04, 11.7, 50.4, 44.8, and 55.3 per cent, respectively, but when compared with the worm removal of the control group (13.1%), there appears to be some evidence of anthelmintic action.

4) Stylomycin was not effective for the removal of Hymenolepis spp. from mice.

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The Hemagglutinating Properties of *Salmonella Typhimurium*

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THE ABILITY of viruses, such as Newcastle disease virus of fowl and influenza virus of man, to agglutinate erythrocytes is a well-known and widely applied phenomenon in the field of virology. Pleuropneumonia-like organisms, associated with the chronic respiratory complex of poultry, have been reported to agglutinate red blood cells, and this property of the organisms has been employed in the development of hemagglutination-inhibition tests for the disease.

Hemagglutination as exhibited by bacterial species has been given less attention than that exhibited by viruses and related microbial forms. Bacterial hemagglutination has been of interest as it related to other intrinsic qualities or added to the basic knowledge of a particular bacterial type.

As early as 1902, Krauss and Ludwig [1] reported the ability of a Vibrio strain and a strain of *Micrococcus pyogenes* var. *aureus* to cause agglutination of erythrocytes. Six years later, Guyot [2] found that 12 of 18 strains of *Escherichia coli* studied agglutinated red blood cells of various animals. Fukuhara,[3] in 1909, demonstrated that organisms belonging to many different bacterial genera agglutinated red blood cells. In 1943, Rosenthal [4] reported that 24 of 70 strains of *Esch. coli* examined agglutinated erythrocytes as well as various other cellular elements such as leukocytes, spermatozoa, yeasts, and mold spores. This phenomenon was thought to be related to a similar hemagglutinating activity found in various bean extracts.

In 1946, Florman [5] associated hemagglutination with bacterial contaminants in certain allantoic fluids, although he was unable to reproduce the phenomenon with pure cultures of the contaminating agents or their filtrates. Keogh *et al.*,[6] in 1947, reported that hemagglutination was demonstrable with strains of *Hemophilus pertussis, Hemophilus parapertussis,* and *Hemophilus bronchisepticus.* Griffitts,[7] in 1948, reported studies of 21 strains of *Shigella alkalescens* which exhibited hemagglutinating properties toward human erythrocytes. He employed the tube-agglutination test for demonstrating the relative development of the hemagglutinating principle in various strains of the organism. Kauffmann,[8] in 1948, in studies of hemagglutination by *Esch. coli,* found no direct connection between the hemolytic and hemagglutinating properties of the strains examined.

Extensive studies of hemagglutination by strains of *Esch. coli* were reported in 1955 by Collier and Jacoeb,[9] Collier *et al.*,[10,11] and Collier and DeMiranda.[12] Collier *et al.*[11] demonstrated that 53 of 105 *Esch. coli* strains studied possessed hemagglutinating properties. Duguid *et al.*,[13] in 1955, reported studies of 47 strains of *Esch. coli,* 34 of which possessed some degree of hemagglutinating activity. Strains capable of causing hemagglutination were found to possess nonflagellar, filamentous appendages (fimbriae) which were demonstrable by electron microscopy. These investigators also observed hemagglutinating activity in certain strains of *Salmonella typhosa, Salmonella enteritidis, Salmonella paratyphi* B, and the paracolons.

During recent investigations of *Salmonella typhimurium* strains for use in the production of rapid whole blood-plate antigens for detection of chronically infected poultry, it was observed that many of the cultures under study consistently caused agglutination of normal red blood cells of chickens and turkeys. This property precluded the use of such cultures in the preparation of antigen suspensions to be employed with whole blood and stimulated studies to find cultures free of the hemagglutinin. During the course of these studies, observations were made regarding some of the fundamental properties of the hemagglutination phenomenon as exhibited by *S. typhimurium,* and these observations are reported in this paper.

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MATERIALS AND METHODS

Twenty-one smooth strains and one rough strain

(P-7) of *S. typhimurium* were selected for these studies. The cultures had been lyophilized in 1954 shortly before first observations of the phenomenon. The early finding that the hemagglutinin tended to disappear from cultures maintained at room temperature made it necessary to refer back to the lyophilized stock from time to time for fresh transplants. Fresh bacterial suspensions were prepared from lyophilized cultures and used within 72 hours for these studies. Suspensions when not in use were stored at 5 C.

Cysteine-glycerin agar,* an experimental medium under study for antigen production, was used to cultivate the organisms. The medium was dispensed as slants in large agar tubes (150 mm. by 16 mm.). Following an incubation period of 24 hours at 37 C., the growth was harvested in 0.85 per cent saline containing 0.5 per cent formalin. The density of each suspension was adjusted to 100 times tube 1 of the McFarland scale,[16] using physiological saline containing 0.5 per cent formalin as the diluent. This suspension is referred to as "undiluted" in the tables which follow. Twofold saline dilutions, ranging from 1:1 to 1:64, were prepared from each undiluted suspension for titration purposes.

Fresh, citrated chicken erythrocytes that had been washed three times in physiological saline and adjusted to a concentration of 30.0 per cent were used as the standard blood cell suspension throughout the studies. The macroscopic plate technique was the method used for demonstrating hemagglutination. The bacterial suspension, 0.04 ml., was placed within a square on a chemically clean glass plate and an equal amount of the red blood cell suspension was added. The mixture was stirred thoroughly with a loop and the plate was rotated. Hemagglutination was demonstrable by a definite clumping of the blood cells within two minutes.

Four strains, namely P-7, P-23, P-26, and P-31, were selected for more detailed study because of their high hemagglutinin content. These strains were used in experiments to determine the effects of heat, chemical agents, cycles of freezing and thawing, and repeated saline washing upon the hemagglutinin.

A constant-temperature water bath was used in the heat-exposure studies. Twofold saline dilutions ranging from undiluted to 1:64 were prepared of each of the four strains and exposed to temperatures of 50, 60, 70, 75, and 80 C. for one minute. Following the exposure period, each dilution was tested for hemagglutinating activity.

In determining the effect of several chemical agents upon the hemagglutinin, cells were harvested in 0.85 per cent saline containing 0.5 per cent formalin (formolized saline), 0.85 per cent saline containing 0.5 per cent phenol (phenolized saline), and 0.85 per cent saline alone (saline). Twofold saline dilutions of each strain ranging from undiluted (100 times tube 1 of the McFarland scale) to 1:64 were tested on the day of harvesting and again after a storage period of 15 days at 5 C. in their respective solutions.

In the freeze-thaw experiments, undiluted suspensions of the bacterial cells in formolized saline were rapidly frozen in a dry ice-alcohol bath and subsequently thawed by holding the tube in flowing tap water. The suspensions were then centrifuged for five minutes in an angle-head centrifuge at 10,000 r.p.m., the supernatant fluid poured off, and the bacteria resuspended in sterile saline. The undiluted supernatant and resuspended cells, diluted for titration, were immediately tested for the presence of hemagglutinin. Each suspension was exposed to the freeze-thaw technique for a total of 25 times.

In an effort to remove the hemagglutinin from the cells, a suspension of each of the four strains was washed with saline for a total of ten serial washings. After each washing, the cells were sedimented by centrifugation at 10,000 r.p.m., resuspended in sterile saline, titrated, and tested separately from the supernatant fluid.

The effect of storage temperature on a formolized suspension of one strain (P-26) was studied after it was observed that the hemagglutinin tended to disappear from agar slant transplants of cultures kept at room temperature. Aliquots of the suspension adjusted to a density of 100 times tube 1 of the McFarland scale were stored in sealed tubes at 37, 30, 5, and −10 C. Samples were removed at periods of 10 days, titrated, and tested for the presence of hemagglutinin.

Formolized saline suspensions of nine strains of *S. typhimurium* were titrated and tested

TABLE 1—The Hemagglutination Titers of 22 Strains of Salmonella Typhimurium with Normal Chicken Erythrocytes

Culture No.	Undiluted	1:1	1:2	1:4	1:8	1:16	1:32	1:64
P-1	—	—	—	—	—	—	—	—
P-2	4	2	1	Tr	—	—	—	—
P-7	4	3	2	1	Tr	—	—	—
P-8	2	1	Tr	Tr	—	—	—	—
P-9	4	3	2	1	—	—	—	—
P-10	2	2	1	Tr	—	—	—	—
P-16	—	—	—	—	—	—	—	—
P-17	1	Tr	—	—	—	—	—	—
P-18	Tr	—	—	—	—	—	—	—
P-19	—	—	—	—	—	—	—	—
P-23	4	3	2	1	Tr	—	—	—
P-24	—	—	—	—	—	—	—	—
P-25	Tr	—	—	—	—	—	—	—
P-26	4	4	4	3	2	1	Tr	—
P-31	4	4	3	2	1	Tr	—	—
104	1	Tr	—	—	—	—	—	—
113	2	1	Tr	—	—	—	—	—
135	2	2	—	—	—	—	—	—
152	4	3	2	1	—	—	—	—
186	2	1	—	—	—	—	—	—
279	3	2	1	Tr	—	—	—	—
324	—	—	—	—	—	—	—	—

— = negative; 1 to 4 = increasing degrees of hemagglutination; Tr = trace of hemagglutination.

* Beef infusion, 1,000 ml.; sodium chloride, 5 Gm.; bacto peptone, 20 Gm.; glycerin U.S.P., 20 ml.; l (+) cysteine hydrochloride, 1 Gm.; and bacto agar, 30 Gm., to a final pH of 7.2.

for hemagglutinating activity against fresh, citrated blood samples of chicken, turkey, human, sheep, cow, horse, rabbit, guinea pig, and swine origin.

RESULTS

Of the 22 strains of *S. typhimurium* included in this study, only five failed to cause hemagglutination of chicken erythrocytes. The remaining 17 strains contained the hemagglutinin in varying degrees (table 1). The majority of the cultures revealed hemagglutination only when used as concentrated suspensions. Four of the strains caused some degree of hemagglutination at a dilution as high as 1:8 and one strain (P-26) as high as 1:32. The rough strain (P-7) demonstrated no discernible difference from the majority of smooth strains.

The destructive effect of heat on the hemagglutinin was evident first at 70 C. (table 2). There was a decided decrease in hemagglutinin titer at 75 C. A temperature of 80 C. for one minute completely destroyed the hemagglutinin activity of the strains of *S. typhimurium* studied.

Cell suspensions stored at 5 C. in formolized saline retained the ability to cause hemagglutination for a much longer period than organisms harvested and stored in either saline alone or phenolized saline (table 3). The titers of all suspensions listed in table 3 were identical on the day of harvesting with those listed for the respective strains in table 1. Formalin actually had a preservative effect on the principle. At the end of 15 days' storage, the cells harvested in formolized saline still retained their original hemagglutinin titer, whereas the phenolized cells demonstrated hemagglutination only to a dilution of 1:1. The effect of saline alone on the hemagglutinin was intermediate between that of phenol and formalin.

TABLE 2—The Effect of Heat upon the Hemagglutinin

Temperature and Culture No.	Undiluted	1:1	1:2	1:4	1:8	1:16	1:32	1:64
50 C.								
P-7	4	3	2	1	Tr	—	—	—
P-23	4	3	2	1	Tr	—	—	—
P-26	4	4	4	3	2	1	Tr	—
P-31	4	4	3	2	1	Tr	—	—
60 C.								
P-7	4	3	2	1	Tr	—	—	—
P-23	4	3	2	1	Tr	—	—	—
P-26	4	4	4	3	2	1	Tr	—
P-31	4	4	3	2	1	Tr	—	—
70 C.								
P-7	4	3	2	Tr	Tr	—	—	—
P-23	4	3	2	Tr	—	—	—	—
P-26	4	4	4	3	1	—	—	—
P-31	4	3	2	Tr	Tr	—	—	—
75 C.								
P-7	1	Tr	Tr	—	—	—	—	—
P-23	2	1	1	Tr	—	—	—	—
P-26	2	2	1	Tr	—	—	—	—
P-31	2	2	1	Tr	Tr	—	—	—
80 C.								
P-7	—	—	—	—	—	—	—	—
P-23	—	—	—	—	—	—	—	—
P-26	—	—	—	—	—	—	—	—
P-31	—	—	—	—	—	—	—	—

— = negative; 1 to 4 = increasing degrees of hemagglutination; Tr = trace of hemagglutination.

TABLE 3—The Effect of Chemical Agents upon the Hemagglutinin after 15 Days' Storage at 5 C.

	Undiluted	1:1	1:2	1:4	1:8	1:16	1:32	1:64
P-7								
Saline	3	2	—	—	—	—	—	—
Formalin	4	3	2	1	Tr	—	—	—
Phenol	1	Tr	—	—	—	—	—	—
P-23								
Saline	3	2	—	—	—	—	—	—
Formalin	4	3	2	1	Tr	—	—	—
Phenol	2	1	Tr	—	—	—	—	—
P-26								
Saline	4	3	1	Tr	—	—	—	—
Formalin	4	4	4	3	2	1	Tr	—
Phenol	2	1	Tr	—	—	—	—	—
P-31								
Saline	3	2	—	—	—	—	—	—
Formalin	4	4	3	2	1	Tr	—	—
Phenol	2	1	Tr	—	—	—	—	—

— = negative; 1 to 4 = increasing degrees of hemagglutination; Tr = trace of hemagglutination.

The freeze-thaw experiments indicated that the hemagglutinin was tightly bound to the bacterial cells. There was no loss in the ability of the bacterial cells to agglutinate erythrocytes after being frozen and thawed 25 consecutive times, nor was the hemagglutinin demonstrable in the supernatant fluid following thawing and centrifugation of the cell suspensions.

Ten saline washings and centrifugations of fresh cell suspensions did not free any demonstrable quantities of the hemagglutinin into the supernatant fluid.

The hemagglutinin was gradually lost upon storage at all temperatures studied (table 4). Freezing the cell suspensions at —10 C. destroyed the hemagglutinin between 30 and 40 days. Suspensions stored at 30 C. and 37 C. still demonstrated hemagglutinating activity after the same

TABLE 4—The Effect of Storage Temperature upon Formolized Suspensions of Strain P-26

	Undiluted	1:1	1:2	1:4	1:8	1:16	1:32	1:64
Day of harvest	4	4	4	3	2	1	Tr	—
10 days								
—10 C.	4	3	3	2	1	—	—	—
5 C.	4	4	4	3	2	1	—	—
30 C.	4	4	4	3	2	1	—	—
37 C.	4	3	2	1	Tr	—	—	—
20 days								
—10 C.	1	Tr	Tr	Tr	—	—	—	—
5 C.	4	4	4	3	2	1	—	—
30 C.	2	1	Tr	Tr	—	—	—	—
37 C.	2	1	Tr	—	—	—	—	—
30 days								
—10 C.	Tr	—	—	—	—	—	—	—
5 C.	4	4	3	2	1	—	—	—
30 C.	2	1	Tr	Tr	—	—	—	—
37 C.	1	Tr	—	—	—	—	—	—
40 days								
—10 C.	—	—	—	—	—	—	—	—
5 C.	4	4	3	2	1	—	—	—
30 C.	1	Tr	—	—	—	—	—	—
37 C.	Tr	—	—	—	—	—	—	—

— = negative; 1 to 4 = increasing degrees of hemagglutination; Tr = trace of hemagglutination.

storage period although to a lesser degree. Suspensions stored at 5 C. best retained the principle.

The hemagglutinin revealed varying degrees of activity toward the erythrocytes of four of nine animal species studied (table 5). Of nine strains of *S. typhimurium* studied, eight exhibited hemagglutination of chicken, turkey, horse, and guinea pig cells. None of the strains hemagglutinated human, sheep, cow, rabbit, or swine cells. More detailed titrations of positive reactions revealed highest titers with turkey erythrocytes, whereas with chicken and horse cells, titers were comparable. Hemagglutinin titers were lower with guinea pig erythrocytes than with those of the turkey, chicken, or horse.

Discussion

The results of these studies indicate that the presence of a bacterial hemagglutinin in cultures of *S. typhimurium* is rather common. In the examination of numerous strains of *Salmonella pullorum* and *Salmonella gallinarum*, the authors have never encountered a similar phenomenon. The occurrence of hemagglutination of chicken erythrocytes with stained pullorum antigen is a rarity and, when it does occur, it is probably due to unexplained properties of the individual blood sample rather than the presence of hemagglutinins in cultures of *S. pullorum* used in antigen production.

The degree of hemagglutinin development in the strains of *S. typhimurium* studied was found to vary widely. Some strains consistently revealed much higher titers of the principle than others when maintained under identical conitions. Other strains never exhibited the phenomenon. No efforts were made to stimulate the development of the hemagglutinin in negative strains. Duguid *et al.*[13] were able to stimulate the development of hemagglutinin in inactive cultures of *Esch. coli* by serially subculturing the organisms under anaerobic or poorly aerobic conditions. No studies of *S. typhimurium* hemagglutinin have been undertaken to relate its presence to the virulence, antigenic qualities, or other basic properties of the organism.

The hemagglutinin of *S. typhimurium* is susceptible to heat and rapidly loses its potency in saline and phenolized saline suspensions. The hemagglutinins of other bacterial genera have also been shown to be susceptible to heat. Dinter and Bakos[15] found that 56 C. for ten minutes destroyed the hemagglutinating principle of *Erysipelothrix rhusiopathiae*, whereas Griffitts[7] reported that 93 C. for one minute was required to destroy the principle in cultures of *S. alkalescens*. Rosenthal[4] found that the hemagglutinin of *Esch. coli* was

TABLE 5—Hemagglutinating Activity of Salmonella Typhimurium with the Erythrocytes of Different Animal Species

Strain	Chicken	Turkey	Man	Sheep	Cow	Horse	Rabbit	Guinea pig	Swine
P-1	—	—	—	—	—	—	—	—	—
P-2	+	+	—	—	—	Tr	—	Tr	—
P-7	+	+	—	—	—	+	—	+	—
P-8	+	+	—	—	—	+	—	+	—
P-10	+	+	—	—	—	+	—	+	—
P-17	+	+	—	—	—	+	—	Tr	—
P-23	+	+	—	—	—	+	—	Tr	—
P-26	+	+	—	—	—	+	—	+	—
P-31	+	+	—	—	—	+	—	+	—

— = negative; + = positive hemagglutination; Tr = trace of hemagglutination.

destroyed when exposed to a temperature of 100 C. for two minutes.

The hemagglutinin of *S. typhimurium* tended to lessen or disappear after several months in cultures maintained at room temperature. The principle is best preserved by lyophilization or storage of cultures at 5 C. It has not been possible to demonstrate any direct relationship between the development of the hemagglutinin and the mediums used for cultivation of the organisms. Other solid mediums studied included beef infusion and K agars.

In preliminary studies, it has been demonstrated that the hemagglutinin can be neutralized by rabbit antiserum containing a mixture of somatic and flagellar *S. typhimurium* agglutinins, whereas normal rabbit serum has no effect upon its activity.

Since efforts to separate the hemagglutinin from *S. typhimurium* were unsuccessful, it is concluded that the principle must be closely bound to the cells. Similarly, Duguid *et al.*[12] found the hemagglutinin of *Esch. coli* to be present on the bacilli and not diffusible because bacilli-free filtrates and supernatants of cultures were inactive. They were able to watch the attachment of bacilli to red blood cells as it occurred in wet films under the light microscope. Those workers suggested that since the organisms tended to agglutinate solid particles other than blood cells, this phenomenon may facilitate the absorption of various nutrients by the organisms during digestive processes in the intestinal tract.

These studies indicate the necessity of carefully selecting strains of *S. typhimurium* for the production of diagnostic antigens that are to be used with whole blood of chickens and turkeys. While proper heating will destroy the hemagglutinin, it may have adverse effects on the antigenic qualities of the organisms. Selection of stable nonhemagglutinating strains of *S. typhimurium* for whole blood-antigen studies is recommended. The organism affords a ready source of hemagglutinin for more basic studies relating to bacterial hemagglutination.

SUMMARY

The ability of *Salmonella typhimurium* to agglutinate erythrocytes of chickens and turkeys, as well as other animal species, is described and discussed. The hemagglutinin was demonstrable in 17 of 22 strains of the organism studied. Hemagglutinating activity was destroyed by heating culture suspensions at a temperature of 80 C. for one minute, and it tended to lessen or disappear in cultures maintained at room temperature. Formalin had a preservative effect upon the hemagglutinin. The principle is apparently closely bound to the bacterial cells and is not diffusible. Efforts to release it by freeze-thaw techniques and saline washing were not successful. The results of this study indicate the necessity of carefully selecting strains of *S. typhimurium* free of hemagglutinating activity for the production of diagnostic antigens that are to be used with whole blood of chickens and turkeys.

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Studies on *Pasteurella Multocida*. III. A Serological Survey of Bovine and Porcine Strains from Various Parts of the World

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FOR SEVERAL DECADES, attempts have been made to develop an adequate serological classification of *Pasteurella multocida*. The reports describing the results of these efforts have been reviewed comprehensively by several authors.[11, 12] In spite of the large amount of effort expended, no satisfactory serological classification evolved.

The demonstration recently of a soluble, specific capsular antigen on strains of *Past. multocida*[2] offered a rational basis for a serological classification. In a recent report,[6] a hemagglutination test was described for the routine identification of the four principal serological types, *viz.* A, B, C, and D. The purposes of this report are: (1) to record some additional observations on the hemagglutination test; (2) to present information on the serological type, geographic source, associated pathology, and dissociation status of bovine and porcine strains of *Past. multocida;* and (3) to make recommendations regarding the serological types to be included in producing various bacterins and antiserums for the prevention of pasteurellosis in cattle and swine.

The term hemorrhagic septicemia has been used in this paper to designate an acute systemic Pasteurella infection of the kind studied by Bain[1] in East Asia. Hemorrhagic septicemia does not appear to have occurred in North America as an epizootic disease in recent years. Shipping fever has been erroneously referred to as hemorrhagic septicemia for many years.

MATERIALS AND METHODS

Strains of Pasteurella Multocida Examined.—Strains of *Past. multocida* were submitted by laboratories located in various parts of the world. Although most cultures were freshly isolated and had undergone few transfers, some were stock cultures of varying ages. The conventional criteria as outlined by Merchant[10] for the identification of *Past. multocida* were employed.

Performance of the Hemagglutination Test.—The hemagglutination test was carried out in precisely the manner described previously.[6] It involved the adsorption of the specific capsular substance, in the form of a saline extract, to type O human erythrocytes and the addition of these treated cells to dilutions of the typing serums. A positive reaction consisted of agglutination or clumping of the red cells after the tubes were left at room temperature for one to two hours.

Four serum dilutions were employed routinely, *viz.* 1:20, 1:40, 1:80, and 1:160. These were the dilutions obtained after the addition of the treated red blood cells. The dilutions prior to the addition of the treated cells were listed in the original report. A positive reaction in the first two dilutions was required for type identification.

It was pointed out previously[6] that strong cross reactions were observed with mucoid cultures when bovine serums were used. These cross reactions were eliminated when carefully prepared rabbit and chicken immune serums were employed.

Acriflavine Test.—The acriflavine test for the identification of antigenic characteristics of colonies of *Past. multocida* has been described.[7] It was observed with this species, as with other bacteria, that antigenic characteristics may change without a corresponding change in colonial morphology. The nomenclature used for the description of dissociation in this study was: for colonial morphology, mucoid and smooth; for antigenic character, M, S, and S^R. The most important type of colony from the standpoint of serology was S^R. The S^R colonies are smooth morphologically but rough antigenically, *i.e.*, they do not possess the specific capsular substance.

All of the cultures examined serologically were subjected to the acriflavine test. The only reaction which has been listed has been the flocculent reaction obtained with cultures composed of mostly S^R colonies. This reaction has been designated in table 3 by a plus sign.

RESULTS

Serological Findings.—In all, 68 bovine strains were typable. Information relative to the occurrence of the different types in relation to geographic origin and disease is given (table 1). It can be seen that three

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This study was aided by a grant from the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y. The authors thank the various laboratories, too numerous to mention, whose contributions of cultures made this study possible. The majority of the Asiatic strains were contributed by R. V. S. Bain of the University of Sydney, Australia.

TABLE 1—Relation Between Serological Type, Disease Produced, and Geographic Origin of 68 Bovine Strains of Pasteurella

No. examined	Source	Disease produced	Serological type
1	Africa	Septicemia	A
1	Africa	Hemorrhagic septicemia	A
1	Alberta	Pneumonia	D
5	Alberta	Pneumonia	A
1	Alberta	Abscess	A
1	Alberta	—	A
1	Australia	Pasteurellosis	B
4	Burma	Hemorrhagic septicemia	B
1	Burma	Hemorrhagic septicemia†	A
3	Ceylon	Hemorrhagic septicemia	B
1	England	Nat. Coll. 3195A*	B
1	Egypt	Hemorrhagic septicemia†	B
1	England	Pneumonia	A
1	England	Hemorrhagic septicemia	A
1	Egypt	Hemorrhagic septicemia	B
4	India	Hemorrhagic septicemia	B
1	Indonesia	Hemorrhagic septicemia	B
1	Iowa	Pneumonia	A
1	Iowa	Encephalitis	D
2	Iran	Hemorrhagic septicemia	B
14	Ontario	Pneumonia	A
1	Ontario	Septicemia	A
1	Ontario	Abscess	A
15	Ontario	Mastitis	A
1	Pakistan	Septicemia	B
1	Philippines	Hemorrhagic septicemia†	B
1	Quebec	Pneumonia	A
1	Thailand	Hemorrhagic septicemia	B

* Culture number in the National Collection of England.

types occur in cattle. Types A and D predominate where North American cattle are concerned. The majority of the strains from the tropical and subtropical countries were type B. Many were recovered from cases of the classic or Asiatic epizootic hemorrhagic septicemia. Most of the Pasteurella strains from cattle in Canada and the United States were associated with pneumonia.

A total of 129 porcine strains were examined. Details relative to these cultures are given (table 2). Although several type B strains were identified, most of the strains from swine were type A and D. The disease with which they were most frequently associated was pneumonia. The picture in regard to the distribution of types would appear to be similar in the major swine-raising districts of the world.

Information relating to the strains that were not typable is also given (table 3). A positive acriflavine test was taken to indicate antigenic roughness or absence of capsular antigen. It is probable that those cultures that were antigenically rough had lost their capsular material as a consequence of a number of transfers on artificial mediums.

DISCUSSION

General Observations.—Observations made during this study indicate that cultures which have been described in the past as mucoid are not necessarily composed of mucoid variants genetically distinct from the smooth variant but rather that the mucoid property is a phenotypic characteristic observed under certain circumstances, e.g., recent isolation, or growth on moist, enriched mediums. As was pointed out previously, strains losing their mucoid character on repeated transfer become smooth from the standpoint of colonial morphology.[7]

The mucoid cultures were successfully typed by the hemagglutination technique. Previously,[8] the capsular substance of mucoid strains was thought to be wholly hyaluronic acid and, therefore, nonantigenic. Further study by the gel diffusion technique[9] has shown that the capsular substance of mucoid strains is a complex and not pure hyaluronic acid. Hemagglutination does not take place if the mucoid capsular substance is exposed to bull's testes

TABLE 2—Relation Between Serological Type, Disease Produced, and Geographic Origin of 129 Porcine Strains of Pasteurella Multocida

No. examined	Source	Disease produced	Serological type
1	Alberta	Lung abscess	D
28	Alberta	Pneumonia	A
23	Alberta	Pneumonia	D
3	Alberta	Rhinitis	D
1	Alberta	Cholecystitis	D
2	Alberta	Septicemia	D
1	Alberta	—	D
3	Australia	Pneumonia	A
1	Australia	—	B
1	California	—	A
1	England	—	D
7	Denmark	Rhinitis	A
9	Denmark	Rhinitis	D
1	France	Pneumonia	B
2	France	Pneumonia	A
1	Georgia	Pneumonia	A
3	Georgia	Pneumonia	D
4	Illinois	Pneumonia	D
1	Illinois	Pneumonia	A
1	Illinois	Hog cholera	A
1	Illinois	Bacteremia	A
3	Iowa	Pneumonia	D
1	Iowa	Pneumonia	A
1	Malaya	Septicemia	B
1	Ontario	Vaginitis	A
1	Ontario	Rhinitis	A
12	Ontario	Pneumonia	A
3	Ontario	Pneumonia	D
1	Wisconsin	Pneumonia	A
5	Wisconsin	Pneumonia	D
1	Quebec	Rhinitis	D
2	Quebec	Pneumonia	A
2	Quebec	Pneumonia	D
1	Quebec	Hemorrhagic septicemia	D

hyaluronidase or a filtrate of a broth culture of *Staphylococcus aureus* prior to exposure to the erythrocytes. All of the markedly mucoid strains have been found to be type A.

It is of interest that no type C strains were identified from either swine or cattle. This serological type has been the most difficult to identify by the hemagglutination test because of the absence or early loss of capsular substance. When the capsular substance is lost, autoagglutination takes place in saline. These antigenically rough strains are agglutinated to high titer with heterologous antiserums. This fact has no doubt accounted for mistakes in the identification of type C strains by workers employing this technique. It is possible that some of the antigenically rough strains (table 3) belonged in the type C category. However, it would appear from observations which will be recorded in the next contribution in this series that most of the type C strains are recovered from cats and dogs.

TABLE 3—Strains of Pasteurella Multocida Not Typable with Typing Serums A, B, C and D

No. examined	Source	Disease produced	Acriflavine
	BOVINE STRAIN		
1	Alberta	—	+
2	Alberta	Pneumonia	+
1	Africa	Pneumonia	+
3	Belgium	Septicemia	+
1	Ontario	Pneumonia	+
1	Ontario	Mastitis	—
	PORCINE STRAIN		
1	Alberta	Pneumonia	—
3	Alberta	Pneumonia	+
1	Alberta	Bacteremia	—
1	Belgium	Septicemia	—
1	California	—	—
3	France	—	+
1	France	Pneumonia	—
1	Illinois	Pneumonia	—

Preparation of Bacterins and Antiserums.—Because the information obtained in this study is based upon the examination of a large number of strains from various parts of the world, it should offer a rational basis for the selection of cultures for the preparation of bacterins and antiserums. There are three principal disease problems in cattle and swine associated with infection with *Past. multocida*. They are: (1) shipping or transit fever of cattle; (2) pasteurellosis (hemorrhagic septicemia) of cattle; and (3) secondary Pasteurella pneumonia of swine. This last-mentioned disease is virus pneumonia of pigs complicated by infection with *Past. multocida*.

Previous studies have shown that *Pasteurella hemolytica* is an important organism in the etiology of shipping fever in Canada.[4,5] A serological examination of strains from animals with shipping fever has shown a complete serological homogeneity.[5] Thus, for the preparation of bacterins and antiserums for the prevention of this disease in Canada, the following cultures should be employed: *Past. hemolytica* and *Past. multocida* type A. Type D should also be considered.

As Bain [1] has suggested, type B or Robert's type I strains should be employed for the production of agents for the prevention of epizootic hemorrhagic septicemia where it occurs.

Pasteurella multocida types A and D should be used for the preparation of bacterins for the prevention of secondary Pasteurella pneumonia of swine.

SUMMARY

A total of 197 cultures of *Pasteurella multocida*, isolated from cattle and swine in various parts of the world, have been serologically typed by the hemagglutination test. There was a predominance of types A and B from cattle. The type A strains were most frequently associated with pneumonia. Type B strains were usually recovered from animals with epizootic hemorrhagic septicemia, for the most part from cattle in tropical and subtropical countries. The type A strains were more prevalent in the countries with a temperate climate.

The strains from swine were predominantly types A and D. Secondary Pasteurella pneumonia was the most important disease condition associated with the isolations from swine.

Recommendations are made in regard to the selection of serological types of *Past. multocida* for the preparation of bacterins and antiserums for the prevention of shipping fever of cattle, hemorrhagic septicemia of cattle, and secondary Pasteurella pneumonia of swine.

Fourteen bovine and porcine strains could not be typed. Of these, seven were

considered nontypable because of antigenic roughness as revealed by the acriflavine test.

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Use of Clearing Agents in Staining Semen Smears

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BASIC STAINS, like crystal-violet, gentian violet, basic fuchsin, etc., are powerful in staining sperm deeply in a short time and in bringing out sperm morphology with remarkable clarity. However, the main difficulty is the intense background staining which obscures sperm morphology. It is, therefore, essential that when the basic stains are employed some chemical reagent should be used to remove the seminal plasma without washing large numbers of sperm from the smears. When semen smears are treated in this manner, the spermatozoa take a brilliant stain and appear with exceptional clarity against the unstained background. Such chemical agents which clear the seminal plasma from the smear are called "clearing agents."

Several methods of clearing semen smears were employed by other workers [1, 2, 5, 7-13] in the past but chlorazene® was the agent most commonly chosen. These investigators used aqueous solutions of chlorazene of various strengths, ranging from 0.5 per cent [12, 13] to a saturated aqueous solution.[5, 9] Similarly, there was also great variation in the period of clearing which ranged from one-half to one minute [9] to an hour.[5, 8] Hotchkiss [3] advocated the use of acid alcohol for clearing smears of human semen. Keaty and Hamblen [4] suggested treatment with water acidified with HCl for removing extracellular debris and mucus from human semen smears. Lane-Roberts *et al.*[6] recommended the use of 0.5 per cent aqueous chloramine or chloramine in Ringer's solution for several minutes for clearing smears of human semen.

This study had two objectives:

A) In view of the great variations in the concentrations of the aqueous solutions of chlorazene employed and also the variations in the periods of clearing employed, it was considered desirable to make a comprehensive study of the efficacy of the various strengths of aqueous chlorazene and to determine the shortest period required for efficient clearing. Similarly, it was also necessary to determine whether any other chemicals would prove satisfactory as clearing agents.

B) Further, instead of clearing the smears first and then staining, it was desirable to ascertain whether both these processes could be conducted simultaneously by mixing the stain and the clearing agent. Such a procedure would reduce considerably the time involved in getting preparations ready for microscopic examination.

MATERIALS AND METHODS

The semen of the bull and the buffalo were used in this study. Smears of both freshly collected and stored semen were employed and no distinction was made between the two. All smears were fixed by simple air-drying. Aqueous solutions of various chemicals of different strengths were employed to determine their suitability as clearing agents. These solutions were always filtered before use. The period of clearing varied from five seconds to 15 minutes, the object being to determine the shortest period within which efficient clearing could be obtained. It was not found necessary to dry the cleared smears after washing before staining. In all cases, only freshly prepared smears or those that were only a few hours old were used.

The cleared smears were stained in either carbol crystal-violet, carbol fuchsin, or a 1.0 per cent aqueous solution of safranin for 15 seconds, and then washed in running tap water and dried by any of the following methods: (1) by keeping the slide in a slanting position underneath a fast-moving fan, or with the air blast from a hair-drier; (2) by keeping the slide on a hot plate at a temperature of about 70 to 80 F.; or (3) by blotting by gently pressing in the folds of a clean blotting paper.

The last-mentioned method of drying was found to give satisfactory results, drying being effected much more quickly.

In the studies on the technique of simultaneous clearing and staining, two separate methods were tried. Details of these techniques are given under each experiment.

A) RESULTS

The results of the studies on the various chemicals as clearing agents for semen

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TABLE 1—Chemicals Suitable as Clearing Agents for Semen Smears

No.	Chemical	Optimum concentration (%, in distilled water)	Clearing period (seconds)
1)	Sodium hydroxide	0.5 to 1.0	5 to 10
2)	Sodium carbonate	1.0 to 2.5	10 to 15
3)	Sodium bicarbonate	1.0 to 2.5	10 to 15
4)	Sodium hexametaphosphate	1.0 to 2.5	10 to 15
5)	Sodium silicate	1.0	10 to 15
6)	Trisodium phosphate	1.0 to 2.5	10 to 15
7)	Disodium phosphate	1.0 to 2.5	10 to 15
8)	Potassium hydroxide	0.5 to 1.0	5 to 10
9)	Potassium carbonate	1.0 to 2.5	10 to 15
10)	Potassium bicarbonate	1.0 to 2.5	10 to 15
11)	Calcium hypochlorite	Saturated aqueous solution	10 to 15
12)	Chloramine T	Saturated aqueous solution	10 to 15
13)	Chlorazene	Saturated aqueous solution	10 to 15

smears are presented below and summarized (table 1).

Sodium Hydroxide and Potassium Hydroxide.—From 0.5 to 4.0 per cent aqueous solutions of sodium or potassium hydroxide were found to give satisfactory results at five to ten seconds' exposure. A 0.5 per cent solution gave results comparable to those obtained with stronger solutions. Two to 4.0 per cent aqueous solutions were powerful and removed a good number of sperm from the smears, whereas at the lower strengths this was not so marked. With 0.25 per cent solution, ten to 15 seconds for clearing are required, while a 0.1 per cent solution required one-half minute for clearing.

Sperm staining after clearing with a 2.0 to 4.0 per cent solution was not as clear or as uniform as that obtained after clearing with the weaker solutions. The sperm heads also appeared much smaller, looking shorter and narrower and the tails also appeared thinner. With 1.0 per cent and weaker solutions, staining was more uniform and satisfactory.

For routine purposes, 0.5 to 1.0 per cent aqueous sodium or potassium hydroxide, five to ten seconds, is recommended for clearing of semen smears.

Sodium Carbonate and Potassium Carbonate.—From 1.0 to 2.5 per cent aqueous solutions of sodium and potassium carbonates were satisfactory at ten to 15 seconds' exposure. Although there was no danger of sperm being washed off with the use of stronger solutions and sperm size or staining were unaffected, the stronger solutions were viscous and required considerable washing to remove the soapy feel of the slides.

Staining of smears cleared in sodium or potassium carbonate was excellent and sperm morphology was clearly brought out. For routine purposes, 1.0 to 2.5 per cent aqueous solutions are satisfactory at ten to 15 seconds' exposure.

Sodium Bicarbonate and Potassium Bicarbonate.—The results obtained with sodium and potassium bicarbonates are similar to those obtained with sodium carbonate. For all practical purposes, 1.0 to 2.5 per cent aqueous solutions are satisfactory at ten to 15 seconds for clearing.

Trisodium Phosphate.—From 1.0 to 2.5 per cent aqueous solutions of trisodium phosphate at 10 to 15 seconds' exposure proved to be most satisfactory. Saturated solution was viscous and not convenient for use.

Disodium Phosphate.—One to 2.5 per cent aqueous solutions of disodium phosphate proved satisfactory for routine purposes at ten to 15 seconds' exposure. The staining results after clearing were excellent.

Sodium Hexametaphosphate.—One to 2.5 per cent aqueous solutions of sodium hexametaphosphate were found to be most convenient for routine use. With solutions stronger than 10.0 per cent, there was danger of sperm being washed from the smear and solutions were also viscous and inconvenient to handle.

Sodium Silicate.—One per cent aqueous solution of sodium silicate was found to give excellent clearing at ten to 15 seconds' exposure. Sperm staining was also uniform and excellent.

Calcium Hypochlorite.—Calcium hypochlorite does not dissolve even to the extent of 0.1 per cent in water in spite of the use of heat. A saturated aqueous solution was, therefore, tried and found to give ex-

cellent clearing after ten to 15 seconds. The staining results were also excellent.

Chloramine T.—Chloramine T also does not dissolve even to the extent of 0.1 per cent in water with the use of heat. A saturated aqueous solution was found to give satisfactory clearing at ten to 15 seconds' exposure. Sperm staining was also good.

Chlorazene.—Various strengths of chlorazene, ranging from 1.0 per cent to saturated aqueous solutions, were tried and clearing periods of ten seconds to seven minutes were employed. The saturated solution and 20.0 per cent solution gave the fastest clearing, requiring ten to 15 seconds without any danger of sperm being washed from the smear. The staining results were also excellent. For all practical purposes, a saturated aqueous solution of chlorazene can be employed at 10 to 15 seconds' exposure.

B) Results

Techniques of Simultaneous Clearing and Staining of Semen Smears.—With the object of determining whether the two separate processes of clearing and staining could be combined in a single operation, the clearing agents listed (table 1) were tried at the lowest concentration specified for each.

The stains used were: carbol crystal-violet, Ziehl's carbol fuchsin, and 1.0 per cent aqueous safranin, these three stains being fairly representative of the basic stains and being some of the most extensively used.

The following two techniques were employed: (1) Smears were flooded with clearing agents followed immediately by the addition of stain, the two being mixed thoroughly by suction with a pipette or dropper followed by the rocking motion of the staining rack. (2) Equal quantities of clearing agent and stain were mixed in a test tube just before staining and the mixture immediately poured over the smear and the slide kept in a rocking motion throughout the staining period.

The duration of staining was determined from the time the stain was added to the clearing agent in the first method and from the time the mixture was poured on the smear in the second method. The clearing agent-stain mixture was then allowed to act for periods varying from five seconds to one minute and then washed in running tap water and quickly dried and examined.

With all the various stains and clearing agents, whenever the two were mixed, there was chemical reaction resulting in the precipitation of the stain and it is, therefore, essential that the slide should be kept in a rocking motion to prevent complete settling of the stain deposits as far as possible. When the two are mixed in a test tube, it is important that it should be done rapidly and the mixture poured immediately over the smear.

When the procedure outlined above was adopted and the precautions mentioned taken, excellent preparations were obtained with practically all the clearing agents and stains that were studied with both the techniques mentioned above. In general, 15 to 20 seconds' exposure resulted in excellent preparations without any background staining and the sperm morphology was clearly delineated. There was no marked difference in the results obtained with each of the two techniques. Studies with other basic stains as well as with other formulas of the three stains mentioned indicate that satisfactory results could be obtained with a wide range of basic stains by these combined techniques.

Effect of Clearing Semen Smears on the Intensity of Sperm Staining.—Clearing agents are generally used in staining semen smears to remove background staining when basic stains are employed. The following experiment was conducted to ascertain whether the clearing agent has any influence on the depth of sperm staining, since that had not been studied.

Smears of several samples of bull semen were prepared and stained, some after clearing and controls without clearing. The following clearing agents and periods were used: (1) saturated aqueous solution of chlorazene, 15 seconds; (2) saturated aqueous solution of calcium hypochlorite, 15 seconds; (3) an aqueous solution (0.1 %) of potassium hydroxide, 15 seconds; and (4) an aqueous solution (1.0 %) of sodium carbonate, 15 seconds.

Only acid stains that do not give rise to any background staining were employed in this study; these included 1.0 per cent aqueous solutions of erythrosin B, merurochrome, aniline blue, and cotton blue. Smears were stained for about one-half minute.

It was found that with the clearing agents tested there was not much difference in the intensity of sperm staining exhibited by cleared and uncleared smears. However, the cleared smears presented a neater background than the control smears and the sperm also took a slightly deeper shade of color. On the whole, there appears to be no advantage in clearing smears before staining with acid stains.

SUMMARY

Studies were made to find new chemicals to serve as clearing agents for semen smears and to determine the most satisfactory concentration and time factor for the clearing process for each. Studies were also made regarding whether the clearing and staining processes could be carried out satisfactorily in a single operation.

The 13 clearing agents tested could be used in conjunction with basic stains successfully to bring about simultaneous clearing and staining of smears with excellent results.

In view of the interaction between the stain and the clearing agent, mixtures of the two have to be prepared fresh each time and can not be stored.

Clearing of semen smears did not materially increase the depth of sperm staining when acid stains were used.

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An Improved Medium for the Cultivation of *Vibrio Fetus*

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A MAJOR PROBLEM confronting investigators studying *Vibrio fetus* is the isolation and cultivation of the organism on commonly used laboratory mediums. Huddleson [7] recommended Difco thiol semisolid medium and indicated it would support growth of *V. fetus* for approximately 150 days at 26 C. This medium has since met with wide approval and is routinely used by many investigators.[3, 4, 11, 13] Other investigators prefer nutrient agar with 5 to 10 per cent defibrinated blood added.[2, 6, 18, 20] Most investigators, however, emphasize that the organism grows better on laboratory mediums after several subcultures and that there is need for a better isolation medium.

As early as 1913, McFaydean and Stockman [10] mentioned a search for a better isolation medium, although their use of an agar-gelatin-serum medium did allow them to do excellent investigations. They found that consecutive transfers in peptone broth would allow the organism to die but, if the transfer was back to broth with agar added, the organism would survive. Pieces of potato added to the peptone broth accomplished the same result as the agar.

Plastridge and Williams [12] obtained no growth on blood agar, liver-infusion agar, or infusion broth to which serum, ascitic fluid, amniotic fluid, or cysteine were added. They preferred a "soft" agar of liver infusion. Plastridge *et al.*[13] later reported the liver-infusion semisolid not as desirable as thiol agar with glutathionine added. For electron microscopy, Rhoades[16] reported satisfactory growth of *V. fetus* on primary isolation using fresh tryptose agar slants. Alexander [1] used a basal medium of Difco brain, liver, and heart and found that the addition of transaminating amino acids or compounds from Krebs cycle gave increased growth and respiration rates. However, Batlin and Wilson [3] found no increase in growth when substituting 16 amino acids for a hydrolyzed casein medium. Recent work, by Osborne and Bourdeau,[11] showed hormones to be of value when incorporated into thiol semisolid medium. They observed increased growth by the addition of corpus luteum extract, progesterone, and testosterone. Workers at the Montana Experiment Station [5] routinely use Huntoon's "hormone" agar and its modifications.

The use of Brucella broth has met with wide approval.[15, 17, 19, 21] With little modification, it will support growth of stock and experimental cultures. Moreover, much of the antigenic work has been done using this medium. In an attempt to enhance growth and eliminate contaminants of *V. fetus* on primary isolation and to preserve the organisms in stock, Schneider and Morse [19] and Kuzdas and Morse [9] have incorporated ox bile with Brucella broth. They reported best results were with 4 to 10 per cent ox bile concentrations.

MATERIALS AND METHODS

Oxygen Utilization Studies.—Conventional manometric procedures were used to study the effect of amniotic fluid on growth of *V. fetus*. *Vibrio fetus*, culture III 289, of ovine origin, was employed because of its recent isolation, stable morphology, and stable growth on laboratory medium. The bovine culture used was an organism recently isolated from an aborted fetus.

Air was employed as the gas phase after it was found that carbon dioxide added to a Warburg vessel had no effect on the growth of *V. fetus* in three hours. Potassium hydroxide was used to absorb any carbon dioxide produced during incubation.

Brucella broth (Albimi®) was used routinely at this station for the cultivation and maintenance of Vibrio organisms and to make comparisons with improved mediums. Cells were grown on agar (2%) slants for 72 hours and harvested in a potassium buffer solution of pH 6.8. The cells were washed, centrifuged twice in the buffer solution, and resuspended. Concentrations of cells as heavy as possible were used. Turbidity readings were made on a Lumetron colorimeter and expressed as optical density. No attempt was made to use a standard number of cells on any particular day because the results were reported as standardized ZO_2 (reported as 10^6 cells).

Ovine amniotic fluid was collected during the

From the Department of Veterinary Science and Bacteriology and published with the approval of the director, Wyoming Agricultural Experiment Station, as journal paper 85.

The data in this paper were obtained as part of Regional Research Project W-27 (vibriosis).

regular kill of a local meat packer.* The fluid was filtered through a Seitz filter and stored at 5 C. Amniotic fluid was aseptically added to Brucella broth after preparation of the sterile medium.

In the bovine amniotic fluid studies, a reconstituted desiccated amniotic fluid (Difco®) was used.

Turbidity Studies.—After noticing a metabolic response of the bovine and ovine isolates to amniotic fluid, it seemed advisable to observe the response of all available laboratory cultures. Growth studies were made using 27 isolates of both bovine and ovine origin. Duplicate observations were made. Brucella broth was used as a control standard to indicate inhibition or stimulation by the improved medium. Initial optical density readings were made after each tube was inoculated with a standard loopful of a 36-hour culture. The tubes were placed in a desiccator jar which was then evacuated to a pressure of 43 cm. of Hg. This was followed by the addition of 10 per cent N_2 by volume. The incubation period was four days at 37 C., after which each tube was mixed thoroughly by shaking, and optical density readings again were made.

* Swift and Co., Scotts Bluff, Neb.

RESULTS

Oxygen Utilization Studies.—A statistical analysis has shown that *V. fetus* cells grown on Brucella broth, with 2 per cent agar and 5 per cent amniotic fluid added, had a significantly higher metabolic rate

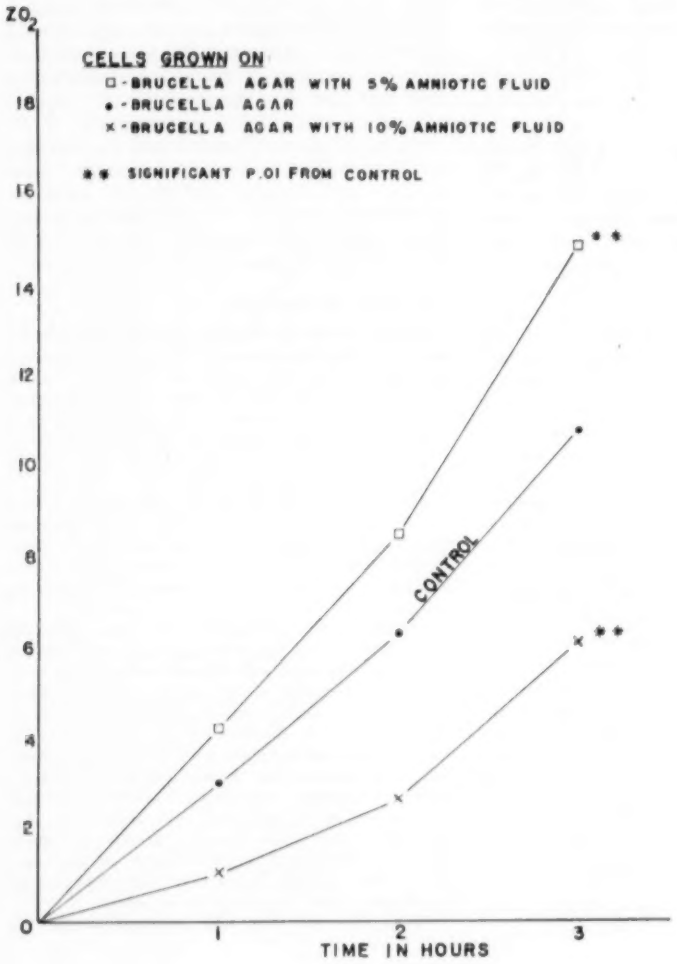

Fig. 1—The average effect observed from five trials on the oxygen utilization of ovine Vibrio fetus isolate III 289 by the addition of 5 and 10 per cent amniotic fluid to Albimi Brucella agar (2%).

than those grown on Brucella broth with agar alone (fig. 1). However, the addition of 10 per cent amniotic fluid significantly inhibited cell respiration.

Desiccated bovine amniotic fluid was used to study the effect on the metabolic activity of both bovine and ovine cultures. The bovine strain (fig. 2) showed a slightly higher metabolic response to the bovine amniotic fluid, but not significantly. When ovine amniotic fluid was used, the ovine isolate showed a growth response greater than the bovine isolate. An analysis of variance showed this response was also not statistically significant.

Turbidity Studies.—Cell growth of 27 isolates of *V. fetus* grown in three different mediums (Brucella broth, Brucella broth with 5 % amniotic fluid, and Brucella broth with 10 % amniotic fluid) was measured after four days of incubation. The "t" test showed the growth of cells in Brucella broth containing 5 per cent amniotic fluid to be significantly higher at the 1 per cent level than the control Brucella broth. Again, the addition of 10 per cent amniotic fluid had an inhibitory effect on the growth of cells.

The 27 isolated organisms were grouped into those of bovine and ovine origin to observe the effect on them of ovine amniotic fluid. The ovine isolates showed a

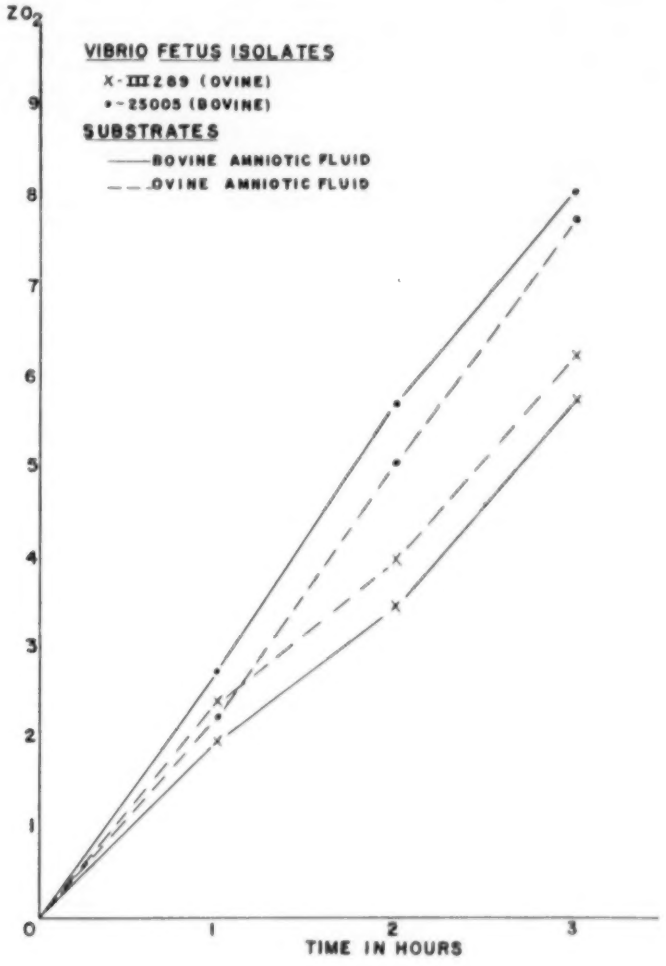

Fig. 2—A comparison of the average metabolic activity of bovine and ovine strains of Vibrio fetus in five replicate trials.

slightly greater response to the ovine amniotic fluid, but the effect was not significant.

SUMMARY

Evidence is presented which indicates that amniotic fluid, when added to Brucella agar (2%) in a concentration of 5 per cent, enhances the growth of *Vibrio fetus*.

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Characteristics of Colonial Forms of *Vibrio Fetus*

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FEW REFERENCES can be found in the literature to critical studies of colonial variation of *Vibrio fetus*.

Many workers, e.g., Smith,[1] Smith and Taylor,[2] Ryff and Lee,[3] Plastridge and Williams,[4, 5] and Hughes,[6] have reported difficulty in obtaining satisfactory growth of *V. fetus* on solid mediums. A film of growth rather than discrete colonies was often described. Even with improved cultural techniques there has been little detailed description of the colonies obtained. Bryner and Frank[7] found two types of growth, discrete colonies and spreading, noncolonial growth. Herzberg and Ristic[8] observed several types of colonial variants when stock cultures of *V. fetus* of human, ovine, and bovine origin were plated on solid mediums. When examined by obliquely transmitted light, smooth, rough, and mucoid colonies could be distinguished, the characteristics of which were similar to those of the variants of Brucella species and *Pasteurella tularensis*.

In recent serological work, there is no mention of the type of colonial growth used in antigen production, indeed the inoculum has usually been taken from semisolid mediums and would thus be likely to contain a mixture of types (Price *et al.*[9] and Biberstein[10]). There is, however, indirect evidence that some strains are nonsmooth; Juhler[11] found that some of his strains agglutinated spontaneously in saline. Biberstein[12] correlated the morphology of the bacterium with antigenic sensitivity, finding that the sensitivity tended to decrease with an increase of coccoid and pleomorphic forms in older cultures. Similarly, Marsh and Firehammer[13] selected smooth cultures on the basis of their bacterial morphology and motility rather than colonial morphology.

The following is a preliminary report of a study of colonial types found to exist within strains of *V. fetus* and the biochemical and serological characteristics of these variants.

MATERIALS AND METHODS

Seventy strains of *V. fetus* from a variety of sources were grown on solid medium and the colonies stained and examined after five to six days' incubation. In a large percentage of the strains, different types of colonies could be easily seen. It was obviously impracticable to work with so many strains so, from this preliminary screening, ten strains were selected for detailed study (table 1). In selecting them, an attempt was made to include strains of bovine, ovine, and human origin; recently isolated strains as well as older stock cultures; and also those strains showing the most distinct colonial differences. Nine of the ten strains selected were catalase positive and H_2S negative; only one strain, number 65, was catalase negative and H_2S positive.

Stock cultures were maintained in Albimi Brucella broth plus 0.1 per cent agar and incubated aerobically. To study the colonial morphology, cultures were grown on Albimi Brucella agar to which an extra 0.5 per cent agar had been added, and plates were incubated in an atmosphere in which the oxygen was reduced to 6.0 per cent by the addition of nitrogen.

Published with the approval of the director of the Wisconsin Agricultural Experiment Station, paper N.S. 219. Supported by the Research Committee of the Graduate School with funds from the Wisconsin Alumni Research Foundation.

Grateful acknowledgment is made by the author to Drs. C. A. Brandly and E. F. McCoy for their advice and suggestions.

TABLE 1—Characteristics of Stock Cultures of Vibrio Fetus Strains

Original designation	Wisconsin laboratory No.	Host	Catalase production	H_2S production	Growth in stab
R.3	14	Bovine	+	—	—
....	70	Bovine (aborted fetus)	+	—	—
....	71	Bovine (aborted fetus)	+	—	—
2029	26	Bovine (fetus)	+	—	—
....	3W	Bovine (aborted fetus)	+	—	—
....	65	Bovine (bull semen)	—	+	+
413A	20	Ovine	+	—	—
543	10	Ovine	+	—	±
1734	61	Human	+	—	—
1154	30	Human	+	—	—

In order to show to best advantage any differences between colonies, the staining technique used by Wilson and White[14] for Brucella was adopted. After five to six days' incubation, those plates showing discrete colonies were flooded with a 1:2,000 solution of crystal-violet for 30 seconds. The stain was poured off and the plates examined under a dissecting microscope, using obliquely transmitted light, as suggested for Brucella by Henry.[15]

The stability of cells in saline was tested by suspending colonies of known type in 0.85 per cent saline and noting the amount of precipitation occurring in 24 hours. Stability in acriflavine was determined by suspending cells in a drop of saline on a slide, adding a drop of 1:1,000 neutral acriflavine, and observing the result under a microscope.

The quantitative catalase test, as described by Bryner and Frank,[16] was used to measure the catalase activity of cultures. The displacement of 5 mm. or more of liquid was regarded as positive. A strip of filter paper impregnated with lead acetate was inserted in the mouth of tubes containing cultures in semisolid medium. Darkening of this strip within 24 to 48 hours indicated a positive H_2S reaction. To test for the reduction of nitrates to nitrites, organisms were grown in semisolid Brucella Albimi medium plus 1 Gm. of sodium nitrate per liter. After three days' incubation, sulfanilic acid and dimethyl-*alpha*-naphthylamine were added to each culture, a pink color indicating the presence of nitrites. The medium for the deep stab growth test was Albimi Brucella broth plus 0.5 per cent agar. Colonies of known type were inoculated into the tubes using a platinum wire and the tubes incubated aerobically. Some cultures grew on the surface and along the line of the inoculum to the bottom of the tube, whereas others grew only on the surface.

For the serological work, it was necessary to produce antigens of pure colonial type. The organisms were grown on Albimi Brucella agar in 12-oz. medicine bottles and incubated in the same reduced oxygen atmosphere. The inoculum was taken from cultures which had been verified as all one colonial type. After four to five days' incubation, the bottles were examined for purity, and material from each bottle plated to determine the types of colonies present. The cells were harvested in saline, filtered through glass wool, washed, and resuspended in 0.3 per cent formol saline. The antigen from each bottle was held separately for four to five days until the plates were examined. Then all antigen which showed no evidence of dissociation was stored in a concentrated form at 4 C. When used, it was diluted to a density giving 70.0 per cent light transmission on an Evelyn photoelectric colorimeter.

Immune serum was produced in rabbits. Serums were first tested to see that there was no nonspecific agglutination. The rabbits were injected with formalized antigen at three- to four-day intervals. Each animal was given five injections, increasing in volume from 0.25 ml. to 1.0 ml. A week after the last injection, the rabbits were bled and the serums frozen until used.

RESULTS

Three main types of colony could be found among the ten strains of *V. fetus* studied. These corresponded closely to the smooth, rough, and mucoid variants described in other groups of bacteria. Differences in appearance of unstained colonies were often very slight but, stained with crystal-violet, they were easily distinguishable. The consistency of the colony, as seen by touching with a loop, was another reliable means of differentiation.

The smooth colonies were circular with an entire edge, domed, fairly opaque, and glistening. When stained, there was an outer purple ring and a clear yellow or yellowish purple center. For the first few minutes after staining, there was a "shimmering" area around the edge of the colony. These colonies were butyrous in consistency, moist, and disintegrated when touched with a loop.

The rough colonies were flatter and duller than the smooth, slightly less regular in shape, and in some strains were papillate. They stained a pale mauve color all over, with no rings of stain or glistening edge. Rough colonies were dry and firm; the entire colony could be moved and, when touched with a loop, it cracked but did not disintegrate.

The third and rarer type of colony was the mucoid. These were most easily distinguished by their viscous consistency; they could be pulled up in a thread with a loop and tended to adhere to the medium. Unstained, the colonies were usually yellowish in the center with a clear outer ring, rather flat, and slightly irregular in shape. They stained heavily, deep purple at the edge with an orange-purple center.

It was essential to examine the plates immediately after staining, as the colonies gradually took up more stain and lost their characteristic appearance. Colonies remained viable for a few hours after staining and could be subcultured during this time.

Morphologically, the three types were identical—typical comma-shaped bacteria existing singly, in pairs, or in short chains. However, on solid medium, the tendency

for an increase in coccoid forms as the culture aged appeared to be more pronounced in smooth variants. Smooth colonies might contain up to 50 per cent of coccoid forms by the third or fourth day of incubation, whereas in mucoid and rough colonies of the same strain, under optimum conditions, no coccoid forms were likely to be found until the fifth or sixth day. Smooth and rough forms were actively motile while some mucoid forms showed a loss of motility (table 2).

The stability of several strains in physiological saline and acriflavine is shown (table 2). Smooth and, to a slightly lesser extent, mucoid colonies could be easily suspended in saline; rough colonies could be suspended only with difficulty and they rapidly precipitated unless previously washed several times in saline. In acriflavine, smooth cells remained in even suspension, rough cells showed granular clumping, and cells from mucoid colonies formed viscous strings.

Stock cultures of the ten strains studied here had been tested several times previously and nine were found to be catalase positive and H_2S negative. Both the rough and smooth colonial types of these strains, when tested separately, were the same, *i.e.*, catalase positive and H_2S negative. There was no significant difference in amount of gas produced by the rough and smooth forms of each strain. Both reduced nitrates to nitrites and failed to grow in deep stab cultures. However, all the mucoid forms isolated from these strains were catalase negative and H_2S positive (table 3). There was definite blackening of the lead acetate paper after 24 hours and only a few bubbles of gas produced in the catalase test. The mucoid types grew well in deep stab cultures along the line of inoculation and reduced nitrates to nitrites. The remaining strain, 65, was previously listed as catalase negative and H_2S positive and, on examination, the stock culture was found to contain nearly 100 per cent mucoid colonies. But from this culture, it was possible to isolate a small number of smooth colonies which, on subculture, were catalase positive and H_2S negative.

TABLE 2—Stability of Colonial Variants of Strains of Vibrio Fetus

Strain	Type	Autoagglutination in saline (0.85%)	Stability in acriflavine	Motility
14	Smooth	—	Stable	+
14	Rough	+	Clumping	+
20	Smooth	—	Stable	+
20	Rough	+	Clumping	+
20	Mucoid	—	Stringy	—
3W	Smooth	—	Stable	+
3W	Rough	+	Clumping	+
3W	Mucoid	—	Stringy	+
70	Smooth	—	Stable	+
70	Rough	+	Clumping	+
70	Mucoid	—	Stringy	—
65	Mucoid	—	Stringy	+

TABLE 3—Biochemical Characteristics of Colonial Variants of Vibrio Fetus

Strain	Type	Catalase production	H_2S production	Growth in deep stab	Reduction of nitrates
20	Smooth	+	—	—	+
20	Rough	+	—	—	+
20	Mucoid	—	+	+	+
70	Smooth	+	—	—	+
70	Rough	+	—	—	+
70	Mucoid	—	+	+	+
3W	Smooth	+	—	—	+
3W	Rough	+	—	—	+
3W	Mucoid	—	+	+	+
65	Mucoid	—	+	+	+

By picking single colonies from plates and continually culturing on well-dried solid medium, these three colonial variants of *V. fetus* could be maintained pure. If, however, single colonies were inoculated into semisolid medium or broth, dissociation occurred. The amount and frequency of this dissociation appeared to be a characteristic of the strain used (table 4). Thus, both the rough and smooth types of strain 61, when grown in semisolid medium or broth and incubated under a variety of conditions, frequently showed dissociation within a few days. Dissociation occurred less often in strains 20 and 30, and strain 10 which existed only in the rough form had never been seen to show any dissociation. This applied to the smooth and rough forms in which mutation occurred in both directions, *i.e.*, S→R and R→S. The mucoid type was found less frequently but, once established, it appeared to outgrow the other two types, e.g., strain 70 mucoid (table 4), and was relatively stable. From one culture of 70 mucoid, however, it was possible to isolate a rough variant, which showed all the characteristics of other rough variants of the strain, *i.e.*, catalase positive, H_2S negative, and unstable in saline.

In semisolid mediums, the smooth form

TABLE 4—Colonial Variation in Semisolid and Liquid Mediums

Strain	Type	No. of cultures examined	No. showing dissociation after 10 days' incubation	Percentage of cultures showing dissociation	Range of percentage of dissociation at 10 days	Av. percentage of dissociation at 10 days
61	Smooth	17	14	82	2 to 100	48
61	Rough	15	10	67	4 to 70	42
10	Rough	8	—	—	—	—
70	Smooth	14	6	43	1 to 100*	41
70	Mucoid	15	1	7	....	34
20	Smooth	15	5	33	1 to 30	9
20	Rough	12	8	66	5 to 98	62
30	Smooth	13	3	23	5 to 50	19
30	Rough	11	2	18	50 to 78	64

* High percentage of dissociation was to the mucoid type; low percentage of dissociation was to the rough type.

grew evenly into the depth of the medium, whereas the rough variant formed a white granular layer at the surface. In broth, the smooth form gave an even turbidity with no deposit, but growth of the rough type was granular with a heavy deposit. In semisolid mediums, the mucoid type grew as a thick, brownish white, viscous, surface layer.

The agglutination reactions of the smooth, rough, and mucoid antigens of several strains of *V. fetus* with antiserums against the smooth variants of these strains are given (table 5). The homologous titer was always the highest but the smooth antigens showed considerable cross agglutination between different bovine strains and also between bovine, ovine, and human strains. With few exceptions, the rough antigens showed the same agglutination reactions as the homologous smooth form but to a lower titer. The mucoid antigens, however, showed no agglutination with the smooth antiserums, even that of the homologous strain.

Antiserum was then prepared against the rough antigens of strains 14 (bovine) and 61 (human). These cultures appeared to be fully antigenic as serum of high titer was easily produced in rabbits. The agglutination reactions of various antigens with these two antiserums are given (table 6). No smooth antigens, even of the homologous strain, were agglutinated by either of the serums. There was cross agglutination between all the rough antigens and antiserums, usually to a higher titer than that shown by these antigens with the corresponding smooth antiserums.

The difference between the antigens of the smooth and rough colonial variants of the same strain was further demonstrated by agglutinin-absorption tests (table 7). For example, in strains 14 and 20, the rough antigen absorbed rough agglutinins from antiserums for smooth variants but did not affect the smooth agglutinins, whereas smooth antigen absorbed both smooth and rough agglutinins. It is anomalous that smooth antigens did not absorb rough agglutinins from antiserum for rough variants, e.g., strains 14 and 61, and further agglutinin-absorption tests

TABLE 5—Agglutination Reactions of Smooth Antiserums, with Antigens of All Three Colonial Variants of Vibrio Fetus

Antigens			Smooth antiserums				
Strain	Origin	Type	14	70	71	61	20
14	Bovine	Smooth	2,560*†	640	640	1,280	2,560
		Rough	640	—	—	320	1,280
70	Bovine	Smooth	80	2,560†	1,280	—	—
		Rough	160	1,280	320	80	—
		Mucoid	—	—	—	—	—
71	Bovine	Smooth	640	1,280	2,560†	—	—
65	Bovine	Mucoid	—	—	—	—	—
61	Human	Smooth	2,560	640	640	2,560†	2,560
		Rough	2,560	320	640	2,560	2,560
20	Ovine	Smooth	2,560	—	—	1,280	5,120†
		Rough	160	—	—	320	1,280
		Mucoid	—	—	—	—	—

* Reciprocal of highest dilution showing definite agglutination.
† No agglutination at a serum dilution of 1:40.

are needed to explain fully the antigenic structure of rough and smooth variants.

Agglutinin-absorption tests suggested that the rough antigens of different strains were identical, even where there was evidence that smooth antigens were not identical (table 8).

TABLE 6—Agglutination Reactions of Rough Antiserums with Antigens of All Three Colonial Variants

Antigens			Rough antiserums			
Strain	Origin	Type	14	Bovine	61	Human
14	Bovine	Smooth	—	(2,560)*	—	(1,280)
		Rough	2,560	(640)	640	(320)
70	Bovine	Smooth	—	(80)	—	(—)
		Rough	1,280	(640)	1,280	(80)
		Mucoid	—	(—)	—	(—)
71	Bovine	Smooth	—	(640)	—	(—)
		Rough	....	....	....	....
61	Human	Smooth	—	(2,560)	—	(2,560)
		Rough	2,560	(2,560)	2,560	(2,560)
20	Ovine	Smooth	—	(2,560)	—	(1,280)
		Rough	640	(160)	640	(320)
		Mucoid	—	(—)	—	(—)

* The figures in parentheses are the titer of the same antigen with the smooth antiserum of that strain.

The mucoid variants failed to agglutinate with smooth or rough antiserums, even of the homologous strain. Rabbits were immunized with the mucoid antigens of strains 70 and 3W and serum was obtained with a high homologous titer. All other mucoid antigens agglutinated with these mucoid antiserums, but no smooth or rough antigens, even of the homologous strain, agglutinated at a dilution of 1:40 (table 9). This indicated a substantial difference in antigenic structure between the mucoid and both the smooth and rough types of homologous strains, a result which might have been expected in view of the marked biochemical differences. The antigens of the four mucoid colonial variants appeared to be similar if not identical.

Discussion

The smooth, rough, and mucoid forms described here had been maintained without any change on solid medium for several weeks during which time they were subcultured every six to seven days and could, therefore, be regarded as stable colonial types. The use of crystal-violet solution to stain the colonies revealed differences which were not visible in unstained cultures. However, variation in stained appearance did occur within each group. The majority of these variations were caused by slight changes in environmental conditions and were not constant on transfer. It was important, therefore, to take into account all factors influencing growth and advisable to examine the consistency of colonies as well as their stained appearance.

A possible fourth variant which had been observed occasionally in cultures of both rough and smooth forms, especially the latter, was the "frosted glass" type of colony. So far this has not been isolated in pure culture, being rather unstable, and its full nature is, therefore, not yet known.

The difference in biochemical characteristics between the mucoid type and the homologous smooth and rough forms was of particular interest. The catalase and H_2S tests are now used as criteria of pathogenicity for strains of *V. fetus* isolated from bull semen, preputial washings, and vaginal mucus of cattle (Bryner and Frank [16] and Lindquist [17]). Catalase-negative and H_2S-positive strains which grow in deep stab cultures are regarded as nonpathogenic for cattle and not true *V. fetus*. The previously described mucoid variants of three catalase-positive strains, two of which (70 and 3W) were origi-

TABLE 7—Antigenic Relationship Between Homologous Rough and Smooth Variants

Antiserum		Absorbed by		Antigens					
Strain	Type	Strain	Type	14S	14R	20S	20R	61S	61R
14	Smooth	.....	.....	2,560*	640	0	0	0	0
14	Smooth	14	Smooth	—	—	0	0	0	0
14	Smooth	14	Rough	2,560	—	0	0	0	0
14	Rough	.....	.....	—	2,560	0	0	0	0
14	Rough	14	Rough	—	—	0	0	0	0
14	Rough	14	Smooth	—	1,280	0	0	0	0
20	Smooth	.....	.....	0	0	2,560	1,280	0	0
20	Smooth	20	Smooth	0	0	—	—	0	0
20	Smooth	20	Rough	0	0	1,280	—	0	0
61	Rough	.....	.....	0	0	0	0	—	2,560
61	Rough	61	Smooth	0	0	0	0	—	2,560

* The reciprocal of the highest dilution showing definite agglutination.

TABLE 8—Antigenic Relationship of Rough Variants of Six Strains of Vibrio Fetus

Serum		Absorbed by		Rough antigens					
Strain	Type	Strain	Type	14	70	26	20	61	30
14	Rough	.....		2,560*	1,280	2,560	640	2,560	640
14	Rough	20	Rough	320†	—	—	—	—	—
14	Rough	61	Rough	320†	—	—	—	—	—
61	Rough	.....		640	1,280	1,280	640	2,560	—
61	Rough	70	Rough	—	—	—	—	—	—
61	Rough	26	Rough	—	—	—	—	—	—

* The reciprocal of the highest dilution showing definite agglutination.
† This may be due to incomplete absorption as the serum was absorbed only once.

nally isolated from aborted fetuses, were identical to these so-called nonpathogenic strains, *i.e.*, they were catalase negative, H_2S positive, and grew in deep stab cultures. These mucoid colonies were stable

TABLE 9—Agglutination Reactions of Mucoid Antiserums with Antigens of All Three Colonial Variants

Antigens			Mucoid antiserums			
Strain	Origin	Type	70	Bovine	3W	Bovine
14	Bovine	Smooth	—	(640)*	....	....
		Rough	—	(—)	....	....
70	Bovine	Smooth	—	(2,560)	—	(160)
		Rough	—	(1,280)	—	(1,280)
		Mucoid	10,240	(—)	640	(—)
65	Bovine	Mucoid	1,280	(—)	640	....
3W	Bovine	Smooth	—	....	—	(2,560)
		Rough	....	....	—	(640)
		Mucoid	1,280	....	1,280	....
61	Human	Smooth	—	(640)	....	....
		Rough	—	(320)	....	....
20	Ovine	Smooth	—	(—)	—	(320)
		Rough	—	(—)	—	(1,280)
		Mucoid	640	(—)	1,280	(—)

* The figures in parentheses are the titer of the same antigen with the smooth antiserum of that strain.

types and had been maintained for several months on solid mediums, during which time the biochemical characteristics remained constant.

Thus, a newly isolated strain of *V. fetus*, tested and found to be catalase negative and H_2S positive, might actually be the mucoid variant of a catalase-positive, H_2S-negative, and fully pathogenic strain. In this case, the isolate could not be disregarded as a cause of infection as there is always the possibility of the smooth or rough form reappearing, especially in animal tissues. One example of this was the stock culture of strain 65, isolated from bull semen and previously classed as catalase negative and H_2S positive. Cultures of this strain were found to be almost 100 per cent mucoid but, from one culture, it was possible to isolate colonies of the typical smooth type which were catalase positive and H_2S negative and should, therefore, be classed with the other strains of true, pathogenic *V. fetus*.

Since this work was started, mucoid variants have been isolated from two other catalase-positive strains, and both variants were catalase negative, H_2S positive, and grew in deep stabs. Three strains recorded as catalase negative were found to be 100 per cent mucoid. Of four freshly isolated strains examined, one was mucoid and three were smooth.

According to Bryner and Frank,[16] the catalase-negative type of Vibrio was occasionally found in conjunction with the catalase-positive type in herds with a history of infertility and, in several instances, both types were isolated from the same individual simultaneously. This would be more easily explainable if both types were, in fact, colonial variants of the same strain rather than different species of Vibrio.

The catalase-negative strains isolated by Bryner and Frank [16] were serologically unrelated to catalase-positive strains. Similarly, the mucoid colonial variants were antigenically distinct, showing no agglutination with homologous smooth and rough antiserums. This could be due to the presence of viscous, capsule-like material around the cells. The smooth and rough colonial variants of these same strains showed definite cross agglutination with other catalase-positive, H_2S-negative strains. The four mucoid types studied in detail appeared to be antigenically similar if not identical.

The degree of antigenic relationship between smooth and rough types can not be fully understood until further agglutinin-absorption tests have been carried out. It appears that smooth cells possess some antigenic factor common to rough cells also, since rough antigens were agglutinated by antiserums for smooth variants. However, in smooth cells, this common component must be masked, possibly by more superficially smooth antigen, since

smooth antigens were not agglutinated by antiserums for the rough variants. Several strains in which the smooth antigens were not identical have been shown to possess a common rough antigen. Therefore, certain antigens of the smooth colonial variants appear to be more specific than those of rough variants.

SUMMARY

1) After a preliminary examination, ten strains of *Vibrio fetus* were selected for a detailed study of their colonial morphology and related characteristics.

2) Three main colonial variants were isolated and maintained in pure culture for several months. These were smooth-, rough-, and mucoid-type colonies, which corresponded closely to these same variants found in other groups of bacteria. They could be most easily distinguished on solid medium by the appearance of the colonies when stained with dilute crystal-violet and by the consistency of the colonies.

3) The rough type varied in that it was unstable in physiological saline and showed rapid autoagglutination. In 1:1,000 acriflavine, rough cells showed granular clumping, smooth cells remained stable, and mucoid cells formed viscous strings.

4) Biochemically, the mucoid form was significantly different. Whereas smooth and rough forms were catalase positive, H_2S negative, and did not grow in deep stab cultures, the mucoid variants of these same strains were catalase negative, H_2S positive, and grew in deep stab cultures. All three variants reduced nitrates to nitrites.

5) Antigenically, the mucoid forms were distinct from the homologous smooth and rough variants, but mucoid types of different strains possessed a common antigen. The rough form showed some cross agglutination with homologous smooth forms but there were distinct differences in antigenic structure between the two. The smooth antigens appeared to be the more specific.

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